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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Estrogens and environmental estrogens were shown to suppress apoptosis in MCF-7 cells and increase expression of Bcl-2. These same compounds were unable to suppress apoptosis of MDA-MB-231 cells suggesting an ER-dependent mechanism of action. Phytoestrogens represent another category of environmental estrogens some of which our results demonstrate possess anti-estrogenic activity and induce apoptosis in MCF-7 cells. To investigate the role of Bcl-2 in TNF resistance by estrogens a Bcl-2 expression vector was transfected into MCF-7 cells and stable clones were established. These clones express higher levels of Bcl-2 as compared to vector transfected MCF-7N/Neo cells. The BCL-2 overexpressing cells exhibited a greater resistance to apoptosis as compared to MCF-7N/Neo cells. However suppression of ER function by the anti-estrogen ICI 182,780 blocked the ability of Bcl-2 to inhibit apoptosis suggesting that Bcl-2 was necessary but not sufficient for suppression of apoptosis by estrogen signaling. Subsequent experiments provided evidence that ER suppression of apoptosis was dependent upon both Bcl-2 expression and regulation of signaling by the mitogen-activated protein kinase cascades. We have identified environmental compounds that function to regulate the apoptotic response of breast carcinoma cells through regulation of MAPK signaling and Bcl-2 expression.

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INTRODUCTION

We examine the effects of estrogens and environmental estrogens on TNF-induced apoptosis in MCF-7 breast carcinoma cells. 17-β-estradiol, o,p'DDT and alachlor were shown to suppress apoptosis in MCF-7 cells and increase expression of Bcl-2. These same compounds were unable to suppress TNF-induced apoptosis of MDA-MB-231 cells suggesting an ER-dependent mechanism of action. Phytoestrogens represent another category of environmental estrogens that have been shown to possess both estrogenic and antiestrogenic activities in a number of systems. Our results suggest that certain anti-estrogenic phytochemicals induce apoptosis in MCF-7 cells that may correlate with the protective effects of certain phytochemicals against the development of breast cancer. These results suggest that the ability of environmental estrogens to regulate apoptosis in hormone dependent cells is dependent upon the estrogenic or antiestrogenic nature of the compounds. To investigate the role of Bcl-2 in TNF resistance by environmental estrogens a Bcl-2 expression vector was transfected into MCF-7 cells and stable clones were established. These MCF-7N/SFFV-BCL-2 clones express higher levels of BCl-2 as compared to vector transfected MCF-7N/Neo cells. The overexpressing MCF-7N/SFFV-BCL-2 cells exhibited a greater resistance to apoptosis as compared to MCF-7N/Neo cells confirming the involvement of Bcl-2 in apoptotic resistance. Suppression of ER function by the anti-estrogen ICI 182,780 blocked the ability of Bcl-2 to inhibit apoptosis suggesting that Bcl-2 was necessary but not sufficient for suppression of apoptosis by estrogen signaling. Subsequent experiments provided evidence that the ability of estrogen and environmental estrogens to block apoptosis was dependent upon both Bcl-2 expression and coordinate regulation of signaling by the mitogen-activated protein kinase cascades. Overall this project has identified hormonally active environmental compounds that function to regulate the apoptotic response of breast carcinoma cells. These studies have identified mechanisms by which these agents influence apoptosis through regulation of MAPK signaling and Bcl-2 expression.

BODY

Work performed during this pre-doctoral training grant generated significant research and project accomplishments. The doctoral training aspects of this grant resulted in defense of the dissertation work of the principle investigator in October 1998. Subsequent appointment to a post-doctoral research position in the laboratory of Dr. John McLachlan (November 1998) allowed further investigation and completion of the specific aims of this grant. Subsequent to this appointment was made to a Research Assistant Professor position in the Department of Pharmacology jointly with the Center for Bioenvironmental Research (April 2000). Finally subsequent to training on this grant the PI applied for tenure-tracked positions and accepted a position as Assistant Professor of Medicine and Surgery, in the Department of Medicine, Section of Hematology & Medical Oncology, with an adjunct appointment in the Department of Surgery at Tulane University School of Medicine (April 2002).

Technical objective one focused on assessing the effects of estrogen and various environmental estrogens on regulation of TNF apoptosis in MCF-7 cells. Initial studies characterizing the sensitivity of MCF-7 cells to apoptotic stimuli and the cell death signaling mechanisms utilized was performed and published (appendix documents #1,3). Subsequent research focused on the ability of specific organochlorine pesticides to suppress TNF-induced apoptosis and apoptosis. We demonstrated that the organochlorine pesticides *o,p'* DDT and alachlor, like 17-β-estradiol, have the ability to suppress tumor necrosis factor alpha (TNF)-induced apoptosis in estrogen receptor (ER) positive MCF-7 breast carcinoma cells. To determine if the anti-apoptotic effects of these chemicals are exclusive to ER positive breast cancer cells studies examining the effects on TNF-induced apoptosis on the ER negative MDA-MB-231 breast cancer cell line demonstrated that these compounds do not possess survival effects on ER negative breast cancer cells (Appendix document #2). The ability of these compounds to suppress apoptosis in MCF-7 cells was correlated with an ER-dependent increase in Bcl-2 expression. Taken together these results demonstrate that estrogenic organochlorine pesticides like *o,p'* DDT and alachlor may partially mimic the primary endogenous estrogen, 17-β-estradiol, and function to suppress apoptosis in ER responsive cells (Appendix document #6).Immunoblot analysis revealed an increase in expression of Bcl-2 with all three compounds which was inhibited by ICI 182,780 treatment demonstrated this increase was specific to the ER pathway. This suggests that one mechanism for survival effects of estrogenic compounds on MCF-7 cells may be through their ability to increase expression of the Bcl-2 gene. Task 4 involving investigation of synergistic effects of environmental estrogens has been dropped from the proposed research based upon the retraction of the 1997 Science paper in which the basis for synergistic estrogenic activity of environmental estrogen was described. The inability of selected organochlorines to suppress apoptosis in ER negative MDA-MB-231 cells further suggests the survival effects of these chemicals is dependent on an intact ER signaling pathway. Based on these initial findings, the ability of the organochlorine environmental estrogens to

increase expression of the pro-survival proto-oncogene Bcl-2 was examined. The assessment of Bcl-2 expression in MCF-7 cells partially completes the tasks under Technical objective 2. Mammalian expression vectors for Bcl-2, Bax, Mcl-1 and Bcl-XL were obtained (Task 8). The ability of estrogens to promote cell survival in ER-positive breast carcinoma cells is linked to a coordinate increase in Bcl-2 expression, an effect that is blocked with the pure anti-oestrogen ICI 182,780. The role of Bcl-2 in MCF-7 cell survival was confirmed by stable overexpression of Bcl-2 which resulted in suppression of apoptosis induced by doxorubicin, paclitaxel and TNF as compared to vector-control cells. The pure anti-oestrogen ICI 182,780 in combination with TNF, doxorubicin or paclitaxel potentiated apoptosis in vector-transfected cells. Interestingly, pre-treatment with ICI 182,780 markedly enhanced chemotherapeutic drug- or TNF-induced apoptosis in Bcl-2 expressing cells, an effect that was correlated with ICI 182,780 induced activation of c-Jun N-terminal kinase (JNK). Our results suggest that the effects of oestrogens/anti-oestrogens on the regulation of apoptosis may involve coordinate activation of signalling events and Bcl-2 expression (appendix document #6).

The specific environmental estrogens used in task one were expanded to investigate members of the phytoestrogen family. Although not proposed in the initial grant the attention received regarding their potential health benefits in regards to prevention of breast cancer in population which consume diets rich in these flavonoid phytoestrogens has prompted us to further examine the effects of these chemicals on apoptosis. Initial studies revealed certain flavonoid phytochemicals possessed potent antiestrogenic effects (Appendix documents #4,5). Based upon our previously identified role of estrogen and organochlorine environmental estrogen effects on apoptosis we wished to determine if anti-estrogenic phytochemicals would possess anti-survival effect and promote apoptosis in ER positive MCF-7 breast carcinoma cells. This additional research changes the focus of the initial technical objective to include a broader range of environmental estrogens to be examined. 4-OH tamoxifen, the clinically utilized antiestrogen, has previously been shown to induce apoptosis at high concentrations (10uM) whereas at concentrations less than 10uM has been shown to be cytostatic. The ability of a clinically used antiestrogen to induce apoptosis suggested that antagonistic environmental estrogens such as certain phytochemicals might also function to promote apoptosis in sensitive cells. We have previously described the ability of selected phytochemicals including apigenin, luteolin, flavone, kaempferide and chalcone to act as anti-estrogens in breast carcinoma cell and in yeast based estrogen responsive reporter gene analysis (appendix document #4). To determine if these compounds regulate apoptosis, MCF-7 cells were treated with various concentrations of the above phytochemicals or the clinically used 4OH-tamoxifen and viability was assessed using the crystal violet method. Consistent with previous results, we observed a dose dependent decrease in cell viability of 4-OH tamoxifen treated MCF-7 cells with concentrations of 10 nM to 10. Both luteolin and chrysin, previously described as estrogen receptor binding dependent antiestrogens (BDA), were

assessed for their effects on proliferation/viability. Both luteolin and chrysin lead to an increased proliferation/viability at 1 uM by three days. These two compounds, however, were cytotoxic in MCF-7 cells at concentrations of 10 uM to 50 uM in a time dependent manner. Flavone, apigenin, kaempferide, previously described as estrogen receptor binding independent antiestrogens (BIA), and chalcone were also examined in MCF-7 cells. At a 1 uM concentration all four compounds possessed relatively limited effects on proliferation with flavone and chalcone stimulating proliferation above control at days 3 and 5 and day 3 respectively. Both flavone and apigenin were cytostatic at 10 uM and exhibited cytotoxic effects at 25 and 50 uM concentrations as early as three days. The effects of chalcone and kaempferide on cell death were more rapid in MCF-7 cells. Both compounds induced a time dependent loss of viability at 10 uM. At higher concentrations (25-50 uM) both chalcone and kaempferide exhibited a rapid cell death apparent as early as 24 hours. To examine if the loss in viability induced by the phytochemicals was due to apoptosis we used DNA fragmentation analysis. MCF-7 cells were treated with phytochemicals at 25 uM concentrations for 72 hours and harvested for DNA fragmentation analysis. Consistent with the viability data, no DNA fragmentation was observed in vehicle (DMSO), 4-OH-tamoxifen treatment (100 nM), or coumestrol (25 uM) treated cells at 72 hours (Fig 3A). TNF-α (10 ng/ml), previously shown to induce DNA fragmentation of MCF-7 cells, resulted in significant DNA fragmentation. Consistent with the viability data at 72hrs: kaempferide (32.4+/- 1.4% viable), flavone (54.4+/-10.6%viable), chrysin (30 +/- 1.2% viable), chalcone (12.2+/-1.4% viable), apigenin (23.3+/-0.2% viable), and luteolin (28.2+/-4.8% viable) (not shown) all at 25 uM resulted in DNA fragmentation at 72 hours. Narigenin, at 25 uM (82.6+/- 4.5% viable) did not induce DNA fragmentation at 72 hours. The ability of these compounds to induce apoptotic DNA fragmentation at 50 uM concentrations at 72 hours was also examined. Coumestrol treatment (67.4+/-1.0% viable) did not result in DNA fragmentation. DNA fragmentation was observed in MCF-7 cells treated with kaempferide (23.2 +/- 7.9 % viable), flavone (53+/- 0.9% viable), chrysin (23.6+/- 1.9% viable), apigenin (21.2+/-2.1% viable) and chalcone (14.9+/-1.0% viable) at 50 uM for 72 hours. Flavone was the only compound in which treatment resulted in DNA fragmentation without a concurrent loss of greater that 65 % viability at 72 hours at either 25 or 50 uM. These studies represent a ongoing project in the lab and are in preparation for submission.

Key Research Accomplishments- All Years

- Completion of Tasks 1-3 in MCF-7 (ER +) and MDA-MB-231 (ER-) cells.
- Expansion of specific aim 1 to include examination of the flavonoid phytochemical environmental estrogens.
- Examination of Bcl-2 expression and effects on apoptosis in MCF-7 cells. (Tasks 5, 6, 8, 9)
- Identified a role for coordinate MAPK regulation and Bcl-2 expression in estrogen-mediated cell survival signaling
- Completion of dissertation research (October 1998)

List of reportable outcomes- All Years

July 1997- **Burow, M.E.**, Tang, Y., Beckman B.S. Effects of environmental estrogens on apoptosis in MCF-7 breast cancer cells. Gordon Research Conference, Hormonal Carcinogenesis,

July 1998- Presentation: Tulane University - Morris F. and Margaret Shaffer Award for Excellence in Research

Oct. 1998- Dissertation Defense: "Molecular Mechanisms of Survival Signaling and Suppression of Apoptosis in Human Breast Carcinoma Cells"

Nov. 1998- Publication of: **Burow, M.E.,** Weldon, C.B., Tang, Y., Navar, G.L., Krajewski S., Reed, J.C., Hammond, T.G., Clejan, S. and Beckman, B.S. Differences in Susceptibility to Tumor Necrosis Factor-α-Induced Apoptosis Among MCF-7 Breast Cancer Cell Variants. *Cancer Research* **58**: 4940-4946 (1998). (appendix document #1)

Nov. 1998- Post-Doctoral fellow position (Laboratory of Dr. John McLachlan)

Nov. 1999- Publication of: **Burow, M.E.,** Tang, Y., Collins-Burow, B.M., Krajewski, S., Reed,J.C., McLachlan, J.A., Beckman, B.S. Effects of environmental estrogens on TNF-mediated apoptosis in MCF-7 breast carcinoma cells. *Carcinogenesis* **20(11)**: 2057-2061, (1999). (appendix document #2)

July 1999- Presentation, Gordon Research Conference, Hormonal Carcinogenesis. Antiestrogenic Activity of Flavonoid Phytochemicals Mediated via c-Jun N-terminal Protein Kinase and p38 Mitogen Activated Protein Kinase Pathways.

Oct. 1999- **Burow, M.E.,** Boue, S., Collins-Burow, B.M., Melnik, L., Duong, B.N., Li, S., Wiese, T., Cleavland, E., McLachlan J.A. Phytochemical glyceollins, isolated from soy, mediate anti-hormonal effects through estrogen receptor alpha and beta. E.Hormone (30th anniversary of the 1st estrogens in the environment meeting).

April 2000- Accepted position as a **Research Assistant Professor** in Department of Pharmacology, Joint appointment in Center for Bioenvironmetnal Research, Tulane University.

April 2000- Submitted grant application: "Coactivator and Corepressor Expression as a Mechansism for Regulation of Apoptosis and Cell Survival in Normal, Immortal and Neoplastic Breast Epithelial Cells" Agency: Department of Defense- U.S.A.M.R.M.C. Type: CONCEPT, (Funded): May 15, 2001-April, 2002; $74,250. The primary objective of this proposal is the determine the function that expression levels of specific coactivator and co-repressor proteins have on regulation of apoptosis and cell survival in normal and malignant breast epithelial cells.

June 2000- Publication of: **Burow, M.E.,** Weldon, C.B., Chiang, T-C., Tang, Y., Collins-Burow B.M., Rolfe, K., Li, S., McLachlan, J.A., Beckman, B.S. Differences in protein kinase C and estrogen receptor α, β expression and signaling correlate with apoptotic sensitivity of MCF-7 breast cancer cell variants. *Int. J. Oncol.* **16**: 1179-1187, (2000). (Appendix document #3)

June 2000 "Effects of Estrogens and Endocrine Disruptors on Suppression of Apoptosis in Normal and Neoplastic Breast Epithelial Cells" Agency: Department of Defense- U.S.A.M.R.M.C.Type: **IDEA**, (NOT FUNDED)

June 2000 submitted grant application: "Cross-talk between PI3K-AKT and the ER: a key permissive role for estrogen in survival of normal and neoplastic breast epithelial cells."Agency: Department of Defense- U.S.A.M.R.M.C.Type: **IDEA**, (NOT FUNDED)

June 2000 "PI3K/AKT crosstalk with ER signaling and cell survival" Agency: NIH-NIDDK (Biochemical Endocrinology, BCE) Type: **R01** DK59389-01 submited June 1, 2000; (SCORED-NOT FUNDED)

Oct. 2000 Grant application submitted: "Regulation of flavonoid-SERMs by Coactivator/Signaling" Agency: NIH-NIEHS (Reproductive Endocrinology, REN) In response to: PA 99-111-Coactivators and Corepressors in Gene Expression. Type: **R01** ES11159-01 (NOT FUNDED)

Dec. 2000- Publication of: Collins-Burow, B.M., **Burow, M.E.,** Duong, B.N., McLachlan, J.A. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. *Nutrition and Cancer.* **38(2)**, 229-244 (2000). (Appendix Document #4)

Mar. 2001- Resubmitted grant application: "PI3K/AKT crosstalk with ER signaling and cell survival" (SCORED-NOT FUNDED)

April 2001- Publication of: **Burow, M.E.,** Boue, S.B., Collins-Burow, B.M., Melnik, L.I., Duong, B.N., Li, S.F., Wiese, T., Cleavland, E., McLachlan J.A. Phytochemical glyceollins, isolated from soy, mediate anti-hormonal effects through estrogen receptor alpha and beta. *J. Clin. Endocrinol. and Metabolism* **86(4)**, 1750-1758, (2001). (Appendix Document #5)

Oct. 2001- Publication of: **Burow, M.E.**, Weldon, C.B., Tang Y., McLachlan, J.A., Beckman, B.S. Oestrogen-mediated suppression of TNF-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. *J. Steroid Biochem. & Mol. Biol.* **78(5)**: 409-418, (2001). (Appendix Document #6)

2001 Publication of two critical reviews of endocrine disruption: McLachlan, J.A., Newbold, R.R., **Burow, M.E.,** Li, S.F. From malformation to molecular mechanisms in the male: three decades of research on endocrine disruption. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **109(4)**: 263-272, (2001). (Appendix Document #7) And Gulledge, C.C., **Burow, M.E.**, McLachlan, J.A. Endocrine disruption in sexual differentiation and puberty: What do pseudohermaphroditic polar bears have to do with the practice of pediatrics? *PEDIATRIC CLINICS OF NORTH AMERICA* **48(5)**: 1223-1240, (2001). (Appendix Document #8)

June 2001 Submitted grant application: "Effects of Estrogens and Endocrine Disrupters on the regulation of apoptosis through ER α/β-MAPK signaling in Breast Epithelial Cells" Department of Defense-U.S.A.M.R.M.C.Type: **IDEA**, (NOT FUNDED).

June 2001 Submitted grant application: "p160 hormone receptor coactivators as targets in the regulation of drug-resistance and apoptosis of breast epithelial cells."Agency: Department of Defense-U.S.A.M.R.M.C.Type: **IDEA**,(NOT FUNDED)

June 2001 Submitted grant application: "Potential therapeutic use of glyceollins (I-III), novel anti-estrogenic flavonoid phytochemicals isolated from soy." Agency: Department of Defense-U.S.A.M.R.M.C. Type: **IDEA**, $442,407 (FUNDED) Using a nude mouse implanted breast carcinoma model, this proposal will investigate the possible chemopreventive and therapeutic activities of glyceollins (1-3), novel antiestrogenic flavonoids identified from elicited soy.

2001-2002 Applied for and was offered positions at both LSU-HSC Department of Pharmacology and Tulane University Department of Medicine.

April 2002- Accepted a position as **Assistant Professor of Medicine and Surgery**, in the Department of Medicine, Section of Hematology & Medical Oncology, with adjunct appointment in Department of Surgery, appointment as a program member in the Tulane Cancer Center, and appointment as Program Investigator in the Center for Bioenvironmental Research, Tulane University Medical School.

CONCLUSION

The research performed under this grant has demonstrated that certain organochlorine pesticides can suppress apoptosis in ER responsive MCF-7 cells but not in the ER negative MDA-MB-231 cells. The ability of these compounds to suppress apoptosis is correlated with enhanced Bcl-2 expression. However while Bcl-2 expression represents a required component for estrogen and environmental estrogen-mediated survival it is not completely sufficient to mediate the survival effects of the estrogen receptor. Our further studies reveal that both the rapid cell signaling effects through members of the mitogen-activated protein kinase-signaling pathway. This observation represents an important finding in understanding the mechanisms by which estrogens exert biological effects on breast carcinoma cells and demonstrates that multiple mechanisms exists by which ER+ breast caner cells can use estrogen-ER signaling to both promote proliferation as well as resist apoptosis induced by exogenous stimuli such as chemotherapeutic drugs. The identification of these pathways also represent potential targets for development of drugs, by which drug-resistant or endocrine therapy resistant tumors could be treated. Further examination of certain anti-estrogenic plant environmental estrogens revealed opposite activity the estrogenic organochlorine pesticides. Our data here demonstrate that the effects of hormonally active environmental agents on apoptotic signaling are dependent upon their estrogenic anti-estrogenic activity. These finding demonstrate that relevant environmental containments that mimic and affect steroid hormone signaling pathways exert significant effects on the survival pathways in breast carcinoma cells.

REFERENCES

See cited appendix manuscripts

APPENDICES

Document #1(7 pages): **Burow, M.E.,** Weldon, C.B., Tang, Y., Navar, G.L., Krajewski S., Reed, J.C., Hammond, T.G., Clejan, S. and Beckman, B.S. Differences in Susceptibility to Tumor Necrosis Factor-α-Induced Apoptosis Among MCF-7 Breast Cancer Cell Variants. *Cancer Research* **58**: 4940-4946 (1998).

Document #2 (5 pages): **Burow, M.E.,** Tang, Y., Collins-Burow, B.M., Krajewski, S., Reed,J.C., McLachlan, J.A., Beckman, B.S. Effects of environmental estrogens on TNF-mediated apoptosis in MCF-7 breast carcinoma cells. *Carcinogenesis* **20(11)**: 2057-2061, (1999).

Document #3 (9 pages): **Burow, M.E.,** Weldon, C.B., Chiang, T-C., Tang, Y., Collins-Burow B.M., Rolfe, K., Li, S., McLachlan, J.A., Beckman, B.S. Differences in protein kinase C and estrogen receptor α, β expression and signaling correlate with apoptotic sensitivity of MCF-7 breast cancer cell variants. *Int. J. Oncol.* **16**: 1179-1187, (2000).

Document #4 (16 pages): Collins-Burow, B.M., **Burow, M.E.,** Duong, B.N., McLachlan, J.A. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. *Nutrition and Cancer.* **38(2)**, 229-244 (2000).

Document #5 (9 pages): **Burow, M.E.,** Boue, S.B., Collins-Burow, B.M., Melnik, L.I., Duong, B.N., Li, S.F., Wiese, T., Cleavland, E., McLachlan J.A. Phytochemical glyceollins, isolated from soy, mediate anti-hormonal effects through estrogen receptor alpha and beta. *J. Clin. Endocrinol. and Metabolism* **86(4)**, 1750-1758, (2001).

Document #6 (10 pages): **Burow, M.E**., Weldon, C.B., Tang Y., McLachlan, J.A., Beckman, B.S. Oestrogen-mediated suppression of TNF-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. *J. Steroid Biochem. & Mol. Biol.* **78(5)**: 409-418, (2001).

Document #7 (10 pages): McLachlan, J.A., Newbold, R.R., **Burow, M.E.,** Li, S.F. From malformation to molecular mechanisms in the male: three decades of research on endocrine disruption. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **109(4)**: 263-272, (2001).

Document #8 (18 pages): Gulledge, C.C., **Burow, M.E.**, McLachlan, J.A. Endocrine disruption in sexual differentiation and puberty: What do pseudohermaphroditic polar bears have to do with the practice of pediatrics? *PEDIATRIC CLINICS OF NORTH AMERICA* **48(5)**: 1223-1240, (2001).

Document #9 (9 Pages): curriculum vitae for Matthew Burow

[CANCER RESEARCH 58, 4940–4946, November 1, 1998]

Differences in Susceptibility to Tumor Necrosis Factor α-induced Apoptosis among MCF-7 Breast Cancer Cell Variants[1]

Matthew E. Burow, Christopher B. Weldon, Yan Tang, Gabriel L. Navar, Stanislaw Krajewski, John C. Reed, Timothy G. Hammond, Sanda Clejan, and Barbara S. Beckman[2]

Molecular and Cellular Biology Program [M. E. B., S. C., B. S. B.], Department of Pharmacology [C. B. W., B. S. B.], Tulane Cancer Center [M. E. B., C. B. W., Y. T., T. G. H., S. C., B. S. B.], Department of Surgery [C. B. W.], Center for Bioenvironmental Research [G. L. N., T. G. H, S. C., B. S. B.], Department of Medicine, Nephrology Section, and Department of Pathology and Laboratory Medicine [S.C.], Tulane University School of Medicine, New Orleans, Louisiana 70112, and The Burnham Institute, La Jolla, California 92037-1062 [S. K., J. C. R.]

ABSTRACT

Widespread use of MCF-7 human breast carcinoma cells as a model system for breast cancer has led to variations in these cells between different laboratories. Although several reports have addressed these differences in terms of proliferation and estrogenic response, variations in sensitivity to apoptosis have not yet been described. Tumor necrosis factor α (TNF-α) has been shown to both induce apoptosis and inhibit proliferation in MCF-7 cells. We observed that TNF-α inhibited proliferation in MCF-7 cell variants from three different laboratories (designated M, L, and N). MCF-7 M cells were resistant to TNF-α-induced apoptosis, whereas MCF-7 L cells were moderately resistant to the effect of TNF-α. A third variant, MCF-7 N, underwent apoptosis when exposed to TNF-α. Analysis of the p55 TNF-α receptor (TNFR) 1 expression revealed the greatest expression in MCF-7 N cells, whereas the MCF-7 L and M cells expressed 89 and 67% of MCF-7 N cell TNFR1 levels, respectively. Ceramide generation occurred in all three variants in response to TNF-α treatment, with MCF-7 N cells expressing the greatest increase. Cleavage of the CPP32/caspase 3 substrate poly(ADP-ribose) was observed in MCF-7 N and L cells as early as 3 and 6 h, respectively, but poly(ADP-ribose) cleavage was not observed in MCF-7 M cells. The delayed protease activation in the L variant may represent the mechanism by which these cells display delayed sensitivity to TNF-α-induced apoptosis. Expression of the Bcl-2, Mcl-1, Bcl-X, Bax, and Bak proteins was analyzed to determine whether the differences in MCF-7 cell sensitivity to apoptosis could be correlated to the differential expression of these proteins. Whereas Bak, Bcl-X, and Mcl-1 levels were identical between variants, the levels of Bcl-2 were 3.5–3.8-fold higher and the levels of Bax were 1.5–1.7-fold lower in the resistant variants (M and L) as compared with those of the sensitive variant (N). Taken together, these results suggest that differences in susceptibility to TNF-α-induced apoptosis among MCF-7 breast cancer cell variants may be explained by differences in TNFR expression, ceramide generation, differential expression of the Bcl-2 family of proteins, and protease activation.

INTRODUCTION

The MCF-7 cell line was established in 1973 from a pleural effusion of a patient with metastatic breast carcinoma who was previously treated with radiation and hormonal therapy (1). Since that time, this cell line has become a model system of ER[3]-positive breast cancers (2). Previous studies suggest that MCF-7 cell line variants possess intrinsic differences in estrogen responsiveness and proliferation rates. Osbourne *et al.* (3) reported that MCF-7 cells obtained from different laboratories varied in proliferation rates, ER and progesterone receptor levels, estrogen and antiestrogen responses, and tumorigenicity. Klotz *et al.* (4) showed that different stocks of MCF-7 cells displayed different levels of variant ER mRNAs, which correlated with their differential response to estrogen stimulation. Different MCF-7 variants tested by Villalobos *et al.* (5) exhibited different responses to 17β-estradiol-induced proliferation and expression of the estrogen-responsive genes *pS2* and *cathepsin D*. These reported variations in MCF-7 cells could potentially lead to contradictory results, depending on the origin of the variant of MCF-7 cells studied.

Apoptosis and apoptotic signaling have recently been examined in MCF-7 cells in response to a number of stimuli including okadaic acid, staurosporine, Fas, retinoic acid, vitamin D analogues, 4-hydroxy-tamoxifen, ceramide analogues, hormone withdrawal, and various chemotherapeutic drugs (6–15). TNF-α is also an effective inhibitor of proliferation and inducer of apoptosis in MCF-7 cells (7, 8, 16, 17). In other studies, MCF-7 cells reportedly responded only weakly to TNF-α (18–21). MCF-7 cells made resistant to TNF-α by continuous passaging in increasing concentrations of TNF-α express decreased levels of TNFR and do not activate SMase or phospholipase A_2 with TNF-α treatment (17). The reported differences in the sensitivity of MCF-7 cells to TNF-α and potentially other apoptotic-inducing agents raised the possibility that variations in MCF-7 cell strains among laboratories may account for these discrepancies.

The effects of TNF-α are mediated through two distinct but related plasma membrane receptors, p55 (TNFR1) and p75 (TNFR2). Both receptors generate distinct biological effects, with the cytotoxic effects of TNF-α being primarily mediated through TNFR1 (22, 23). Although these receptors share limited cytoplasmic homology, they activate some overlapping signaling cascades, such as nuclear factor κB, via the recruitment of specific signaling intermediates to the cytoplasmic domains (22, 23). In the case of TNFR1, TNF-α ligation results in association with TRADD (24), which then recruits TNFR-associated protein 2, receptor-interacting protein (25), and FADD/MORT1 (26), association with the receptor is followed by the association of FLICE/MACH1 with the TNFR1 complex (27, 28). Subsequent to the formation of this protein complex, the activation of several signaling cascades including phospholipase A_2, SMase, nuclear factor κB, stress-activated protein kinases, and apoptotic proteases occurs (22, 23). Activation of SMase, resulting in ceramide formation, represents an early event in the apoptotic signaling cascade (29, 30). MCF-7 cells have been shown to activate SMase in response to TNF-α and undergo apoptosis when exposed to water-soluble ceramide analogues (17). Additionally, studies of acidic SMase knockout mice have shown that ceramide generation may be required for apoptosis by TNF-α and other inducers in some cell types (31).

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[3] The abbreviations used are: ER, estrogen receptor; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; PARP, poly(ADP-ribose) polymerase; SMase, sphingomyelinase; TRADD, TNFR1-associated death domain protein; FADD/MORT1, Fas-associated death domain; FLICE, FADD-like ICE; MACH, Mort1-associated CED-3 homologue; ICE, interleukin 1β-converting enzyme; FBS, fetal bovine serum; DAG, diacylgycerol; MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

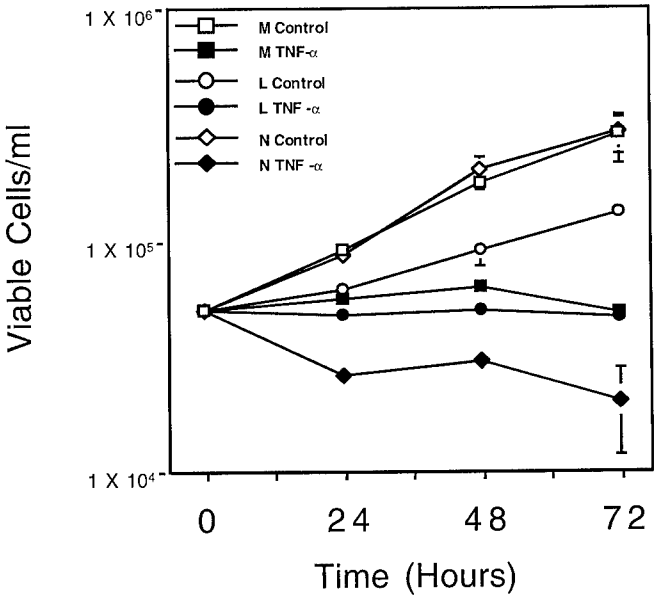

Fig. 1. Effects of TNF-α on the proliferation of MCF-7 cell variants. Each MCF-7 cell variant was plated in DMEM with 10% FBS alone or in the presence of 10 ng/ml TNF-α. Cells were harvested and counted at 24, 48, and 72 h to determine the number of viable cells/milliliter. *Error bars,* SD for duplicate experiments performed in triplicate.

Caspase activation is also thought to represent an early event in TNF-α cell death signaling. The death domain-containing protein FLICE/MACH1/caspase 8 possesses an ICE-like protease domain that becomes activated upon association with the TNFR1-TRADD-FADD complex. The activation of FLICE is thought to result in the subsequent activation of ICE/caspase 1 and CPP32/caspase 3 (32, 33). Therefore, both ceramide generation and protease activation represent potential regulatory points of TNF-α-induced apoptotic signaling.

The Bcl-2 family of proteins comprises a number of related proteins whose expression has been shown to regulate apoptosis (34, 35). This family includes antiapoptotic members (Bcl-2, Mcl-1, and Bcl-X_L) and proapoptotic members (Bax, Bcl-X_S, and Bak) whose individual expression and heterodimerization with each other is believed to regulate the sensitivity of cells to apoptosis. Although the actual biochemical function of these proteins has yet to be completely elucidated, these proteins act upstream of caspase activation through the regulation of cytochrome *c* release from the mitochondria (36, 37).

This study tests directly whether variants in the phenotype of MCF-7 cells may explain the reported differences in susceptibility to apoptosis induced by TNF-α and other agents. The molecular mechanisms for these observations are dissected by examining several events in the signal transduction cascade of TNF-α including TNFR expression and SMase and caspase activation as well as the expression of specific members of the Bcl-2 family of proteins.

MATERIALS AND METHODS

Cell Culture. MCF-7 cell variants M and L were a gift from Stephen M. Hill (Tulane University, New Orleans, LA). The MCF-7 M cell variant (passage 180) originated from the laboratory of the late William McGuire (University of Texas, San Antionio, TX.). The MCF-7 L cell variant (passage 40) originated from the laboratory of Marc Lippman (Georgetown University, Washington, DC). The MCF-7 N cell variant (passage 50) is a subclone of MCF-7 cells from the American Type Culture Collection (Rockville, MD) that was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN). All MCF-7 cells were routinely maintained and grown in DMEM supplemented with 10% FBS, BME (Basal Medium Eagle) amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD), and 1×10^{-10} M porcine insulin (Sigma Chemical Co., St. Louis, MO) under *Mycoplasma*-free conditions.

Proliferation and Viability Assay. MCF-7 cells were plated at 5.0×10^4 cells/ml in 10-cm^2 wells. The cells were allowed to adhere for 18 h before treatment with recombinant human TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN). Cells were then counted at 24, 48, and 72 h posttreatment. The results are represented as the number of viable cells/milliliter as measured by trypan blue exclusion. Apoptosis was expressed as the percentage of trypan blue-stained cells in treated samples compared to control viability. The MTS viability assay (Promega) was performed according to the manufacturer's protocol.

DNA Fragmentation Analysis. After treatment, cells were harvested for DNA as described previously (38). Briefly, $1–2 \times 10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, and 0.5% SDS (w/v; pH 7.4)] to which RNase A (100 μg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added, and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration, 1 M), and lysates were incubated overnight at 4°C. Lysates were centrifuged at $15,000 \times g$ for 30 min, and nucleic acids in the supernatant were precipitated in 2 volumes of ethanol with 50 mM sodium acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Western Blot Analysis. MCF-7 cells were grown for 2 days as described above, and then 5×10^6 cells were harvested in sonicating buffer [62.5 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, and 25 mg/ml aprotinin] and sonicated for 30 s. After centrifugation at $1,000 \times g$ for 20 min, 50 μg of protein were resuspended in sample loading buffer [62.5 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue], boiled for 3 min, and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with a 0.05% PBS-Tween-5% low-fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with rabbit antisera (anti-Bcl-2, 1:4,000 dilution; anti-Bax, 1:4,000 dilution; anti-Bcl-X, 1:1,500 dilution; anti-Mcl-1, 1:2,000 dilution; and anti-Bak, 1:1,000 dilution) or with mouse anti-PARP-specific monoclonal antibody (1:5,000 dilution; PharMingen, San Diego, CA) and incubated for 2 h at room temperature. Blots were washed in PBS-Tween solution and incubated with goat antirabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat antimouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Oxford) for 30 min at room temperature. After four washes with PBS-Tween solution, immunoreactive proteins were detected using the

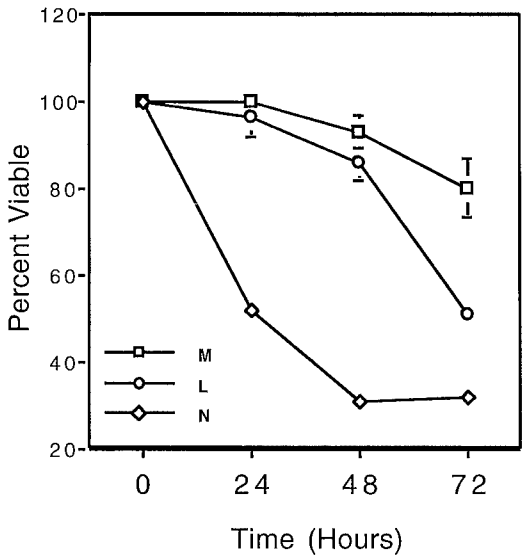

Fig. 2. Effects of TNF-α on MCF-7 cell viability. The percentage of cell death as measured by trypan blue staining at 24, 48, and 72 h of TNF-α treatment in MCF-7 cell variants M, L, and N is shown. *Error bars,* SD for three experiments. An absence of error bars represents <3% error between replicates.

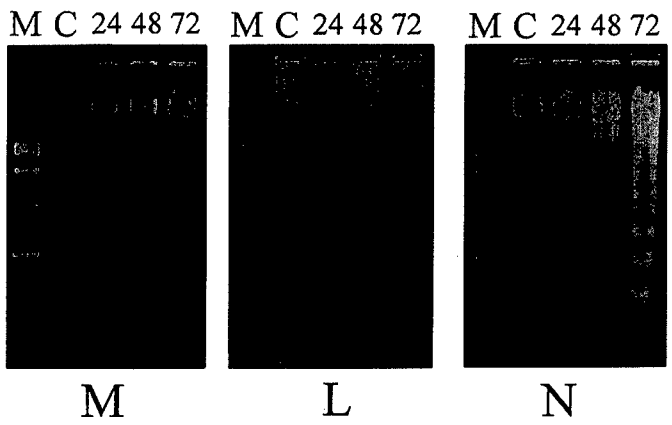

Fig. 3. TNF-α-induced DNA fragmentation of MCF-7 cells. An analysis of DNA fragmentation of MCF-7 cell variants M, L, and N by agarose gel (1.5%) electrophoresis at 24, 48, and 72 h after treatment with TNF-α (10 ng/ml) is shown.

enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad).

Flow Cytometry. Flow cytometric analysis of TNFR1 and TNFR2 was performed as described by Cai *et al.* (17). Briefly, 1×10^6 cells were harvested in PBS-EDTA and washed in 50% normal goat serum at 4°C for 15 min. Cells were washed in PBS-FBS (PBS with 1% FBS added) and incubated with 10 μg/ml mouse anti-TNF-α receptor antibodies (anti-TNFRp55 htr-9 and anti-TNFRp75 utr-1; BACHEM, Inc., King of Prussia, PA) in PBS-FBS at 4°C for 60 min. After this, the cells were washed three times in PBS-FBS and incubated with PE-conjugated goat antimouse IgG (1:40 dilution) in PBS-FBS at 4°C for 2 h. Cells were washed three times in PBS-FBS and analyzed using a Becton Dickinson FACStar flow cytometer. Excitation was at 488 nm (100 mW) using a coherent 6W argon-ion laser. For each cell, emission was measured using a photomultiplier with a 585 ± 42-nm band pass filter for phycoerythrin. Data were collected as 2,000 event list mode files and analyzed using LYSIS II (Becton Dickinson) software. Data represent duplicate counts of 2×10^3 cells, and statistical comparisons were made by Kolmogorov-Smirnov summation curves (39). Background fluorescence was determined using either unstained cells or cells stained using nonspecific mouse anti-α p65 monoclonal antibody (a kind gift of Kathleen Buckley, Department of Neuroscience, Harvard Medical School, Boston, MA).

Fluorescence Microscopy. For fluorescence microscopy, MCF-7 cells were seeded at 1×10^5 cells/ml in 10-cm^2 wells and treated with TNF-α (10 ng/ml) for 48 h. Samples were harvested, pelleted, and fixed in a solution of 10% formalin for 10 min and then washed with PBS and resuspended in a solution of propidium iodide in PBS (50 μg/ml). Cells were transferred to slides and visualized using a Zeiss Axioscope fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with appropriate filters.

Analysis of Ceramide. Ceramide was quantified by the DAG kinase assay as ^{32}P incorporated on the phosphorylation of ceramide to ceramide-1-phosphate by DAG kinase as described previously (40). Briefly, MCF-7 cells were treated with or without TNF-α (10 ng/ml) for the times indicated, washed in PBS, and fixed in ice-cold methanol. After extraction of the lipid, ceramide contained within the organic phase extract was resuspended in 20 μl of 7.5% α-octyl-β-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylenetriamine pentaacetic acid (Sigma Chemical Co.). Thereafter, 40 μl of purified DAG kinase in enzyme buffer [20 mM Tris-HCl, 10 mM DTT, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol (pH 7.4)] were added to the organic phase extract. Ten mM [γ-^{32}P]ATP (20 μl; 1000 dpm/pmol) in a buffer was added to start the reaction. After 30 min at 22°C, the reaction was stopped by the extraction of lipids with 1 ml of chloroform:methanol:hydrochloric acid (100:100:1, v/v). Buffered saline solution [170 μl; 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES (pH 7.2)] and 30 μl of 100 mM EDTA were added. The lower organic phase was dried under N_2. Ceramide-1-phosphate was resolved by TLC using $CHCl_3$:CH_3OH:acetic acid (65:15:5, v/v) as a solvent and detected by autoradiography, and the incorporated ^{32}P was quantified by a phosphorimager (Fugi BAS1000; Fugi Medical Systems). The level of ceramide was determined by comparison to a concomitantly run standard curve composed of known amounts of ceramide.

RESULTS

Using three MCF-7 cell variants (M, L, and N) from established laboratories, we compared the effect of TNF-α on proliferation and viability. Under control conditions, different basal proliferation rates were observed among cell variants with doubling times of 30.8, 45, and 28.6 h for the M, L, and N cells, respectively (Fig. 1). The addition of TNF-α (10 ng/ml) to the medium inhibited basal proliferation in all three variants in a time-dependent manner. The most striking effect was observed in MCF-7 N cells, in which the number of viable cells per milliliter decreased by 50% from that of the control by 24 h. In the TNF-α-treated samples, trypan blue staining indicated that MCF-7 M cells retained >90% viability compared to that of the control on days 1 and 2 and 80% viability on day 3, whereas the viability of the TNF-α-treated MCF-7 N cells was 52, 31, and 32% of the control on days 1, 2, and 3. MCF-7 L cells treated with TNF-α were 97, 86, and 51% viable on days 1, 2, and 3 (Fig. 2). Additionally, the MTS viability assay revealed a dose-dependent effect of TNF-α (0.1–10 ng/ml) on MCF-7 cell variant viability and proliferation (data not shown). Consistent with the literature, 10 ng/ml was the optimal dose for both the induction of cell death (MCF-7 N cells) and the inhibition of proliferation (MCF-7 M and L cells; Ref. 17). These results suggest that MCF-7 N cells are highly sensitive to TNF-α-induced cytotoxic effects. Whereas the MCF-7 M cells were resistant to the cytotoxic effect of TNF-α, they still retained their sensitivity to the antiproliferative effect of TNF-α. TNF-α treatment of MCF-7 L cells resulted in an inhibition of proliferation and a delayed cytotoxic effect.

To determine whether the rapid loss of viability in the MCF-7 N variant on TNF-α treatment was due to apoptosis, DNA fragmentation analysis was performed. As expected from their retention of viability, the MCF-7 M variant did not undergo apoptosis in response to TNF-α

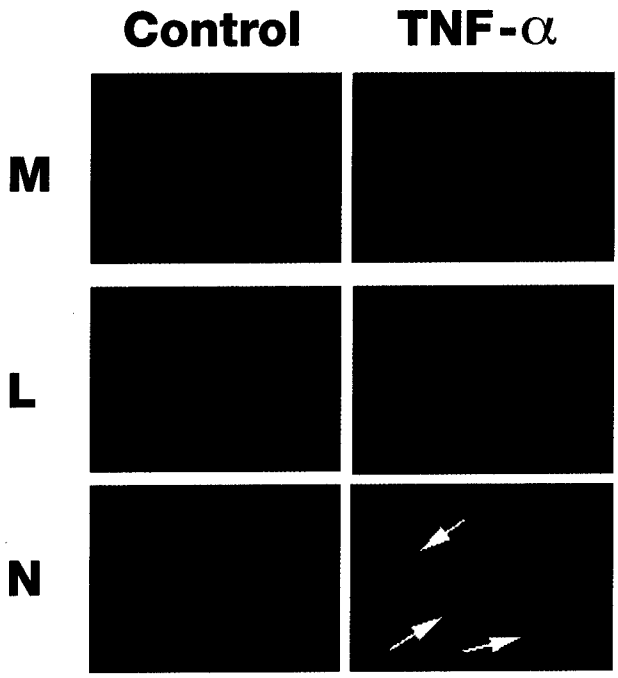

Fig. 4. TNF-α induced apoptosis of MCF-7 cells. Nuclear staining with 50 μg/ml propidium iodide revealed distinct nuclear condensation of the MCF-7 N variant (*arrows*) but not the M and L variants after 48 h of treatment with 10 ng/ml TNF-α.

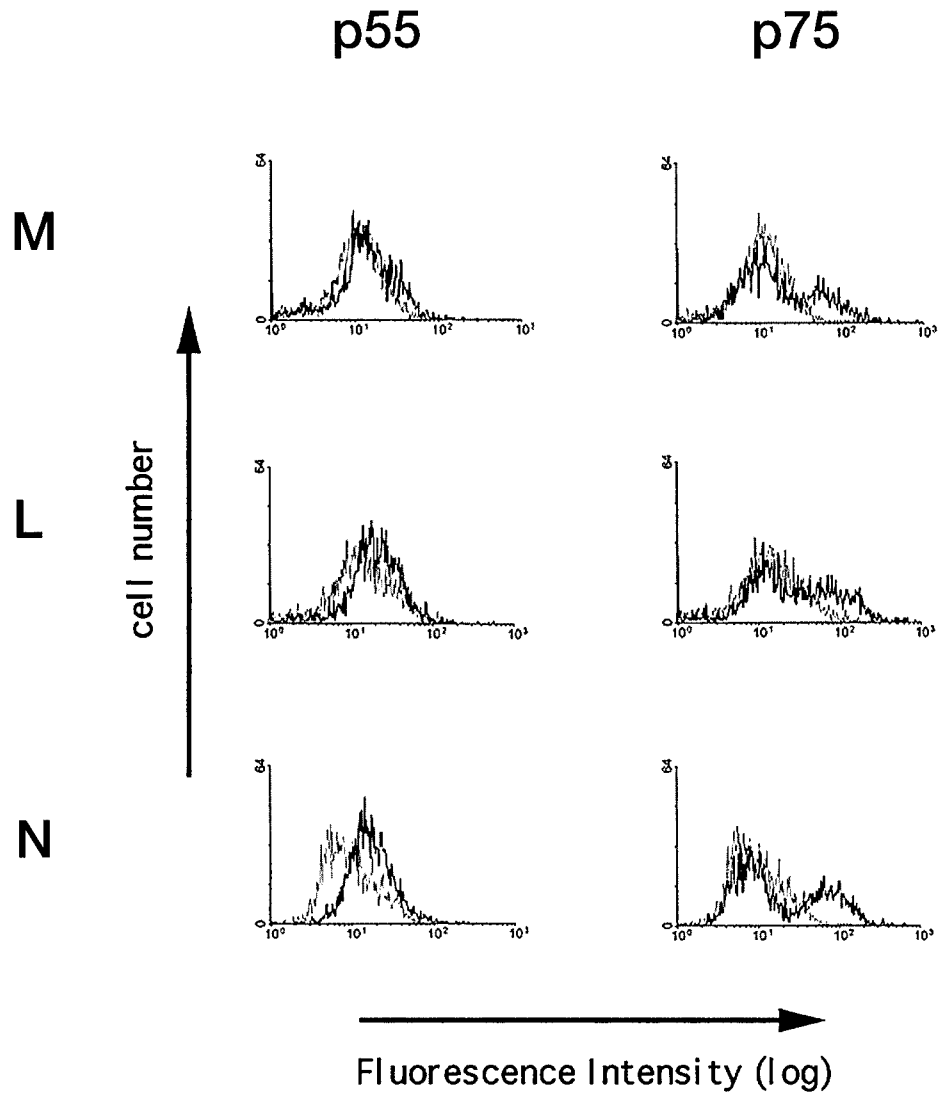

Fig. 5. Analysis of TNFR expression in MCF-7 cells. MCF-7 M, L, and N cells (1×10^6) were detached in PBS-EDTA, washed three times in PBS, and stained with antibodies specific to TNFR1 (p55) and TNFR2 (p75), respectively (*dark lines*), or with nonspecific antibodies to α-p65 (*gray lines*). Each panel is a frequency histogram depicting measurements on 2000 individual cells.

treatment (Fig. 3). However, TNF-α treatment resulted in DNA fragmentation as early as 24 h in the MCF-7 N variant and in moderate DNA fragmentation in the MCF-7 L stock by 72 h. These differences were confirmed by fluorescence microscopy (Fig. 4). Condensed nuclei were observed in TNF-α-treated MCF-7 N cells at 48 h and, as expected, were absent in the MCF-7 M and L variants.

TNFR expression was analyzed by flow cytometry with antibodies specific for TNFR1 (p55) or TNFR2 (p75). In Fig. 5, each curve is a frequency histogram of measurements on 2000 individual cells, with the number of cells in each of 1024 fluorescence channels displayed on a log scale. In each panel, the histogram obtained with the specific p55 or p75 antibody (*dark lines*) is overlaid with the curve derived from a nonspecific antibody to an irrelevant protein (*gray lines*). TNFR1 expression was determined to be 89 and 67% lower in the MCF-7 L and M cells as compared with that in the N cells. This finding suggests that the resistance of MCF-7 M and L cells may be due to their decreased expression of TNFR1. All three cell variants expressed similar levels of TNFR2 (*p75*).

Ceramide generation represents an early downstream event of TNF-α-induced signaling in numerous cell lines including MCF-7 cells (17, 29, 30). Ceramide also represents a key intermediate in the transduction of apoptotic signals from TNF-α as well as Fas, chemotherapeutic drugs, and γ-radiation (29, 30). The ability of water-soluble analogues of ceramide to induce apoptosis in MCF-7 cells further implicates ceramide as an important component in apoptotic signaling. TNF-α-induced ceramide generation was analyzed in the three MCF-7 cell variants to determine whether differences in SMase activity can account for the differential sensitivity to TNF-α-induced apoptosis. A rapid and transient increase in ceramide production was observed in the MCF-7 N variant, reaching a maximal level of 5.5 ± 0.56-fold over that of the control at 15 min with TNF-α (Fig. 6), whereas a 1.73 ± 0.37- and 1.42 ± 0.22-fold maximal increase in ceramide levels was observed in the M and L variants, respectively, at 15 min. All three cell variants possessed similar basal amounts of ceramide. Despite minimal expression of TNFR1, the MCF-7 M and L cells still responded, albeit weakly, to the ability of TNF-α to generate ceramide. Although substantial differences in TNFR1 expression exist between the MCF-7 M, L, and N cells, all cell variants express some degree of TNFR1, which suggests that other downstream events may also account for altered sensitivity to apoptosis.

The caspase family of proteases represents critical signaling intermediates and effectors of the apoptotic program (23, 32, 33). PARP is a proteolytic substrate for Asp-Glu-Val-Asp (DEVD)-specific caspases including CPP32/caspase 3 and caspase 7. Cleavage of PARP from its M_r 116,000 precursor to its M_r 29,000 and M_r 85,000 subunits is indicative of apoptosis and is a useful tool for the measurement of the time course of caspase activity (32, 33). Western blot analysis revealed caspase activity as early as 3 h in the TNF-α-

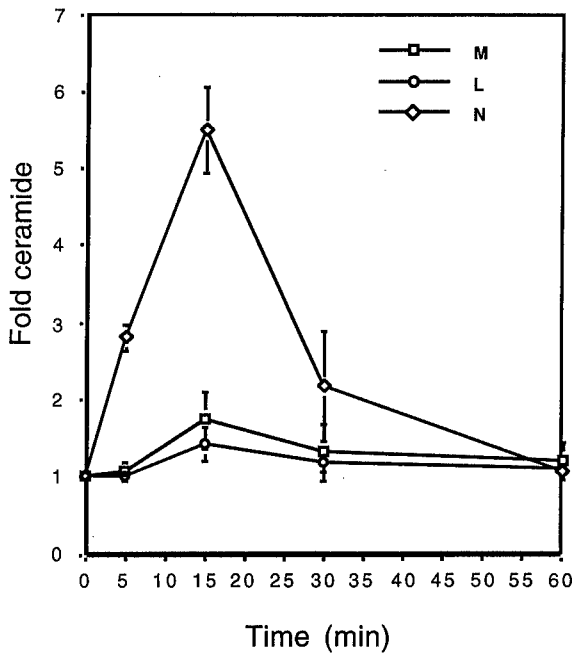

Fig. 6. Ceramide generation in MCF-7 cell variants with TNF-α treatment. MCF-7 M, L, and N cells (4×10^6) were treated with TNF-α (10 ng/ml) for the times shown above. Cells were harvested in ice-cold methanol; the lipid extraction and ceramide assay were performed as described in "Materials and Methods." The ceramide generated represents the fold change over control in nanograms of ceramide/milligrams of protein; *error bars,* SD of three independent experiments performed in duplicate.

sensitive N cells (Fig. 7). PARP cleavage in the moderately TNF-α-sensitive MCF-7 L cells was observed only at 6 h and was not observed in MCF-7 M cells.

bcl-2 proto-oncogene expression imparts considerable resistance to apoptosis induced by a variety of stimuli (34, 35). The relative expression of various members of the Bcl-2 family of proteins was analyzed in the three MCF-7 stocks by Western blot analysis (Fig. 8). Bcl-X, Mcl-1, and Bak protein expression was not appreciably different in the three stocks. However, striking differences were observed in the expression of Bcl-2 and Bax. Bcl-2 expression was 3.8 and 3.5 times higher in the apoptosis-resistant cell variants MCF-7 M and L, respectively, as compared to that in MCF-7 N. Bax expression was found to be 1.7- and 1.5-fold higher in the apoptosis-sensitive MCF-7 N variant as compared to that in the MCF-7 M and L variants.

DISCUSSION

Reported discrepancies exist concerning the apoptotic responses of MCF-7 cells to TNF-α and anti-Fas antibody treatment. Several studies have indicated that MCF-7 cells readily undergo apoptosis in response to TNF-α and anti-Fas (7, 8, 17). However, some reports have indicated that TNF-α and Fas only weakly induce apoptosis in MCF-7 cells (18–21). Others have shown that the cytotoxic *versus* the cytostatic effects of TNF-α depend on the media and serum conditions used to culture the MCF-7 cells (41). We report that under identical culture conditions, variations in apoptotic responses exist among three different MCF-7 cell strains obtained from established laboratories (M, L, and N). It was determined that proliferation of all three variants was inhibited by TNF-α, with the cell number of the MCF-7 N variant decreasing below control in parallel with a decrease in viability. The loss of viability in TNF-α-treated MCF-7 N cells was due to an induction of apoptosis observed as early as 24 h, whereas the MCF-7 L cells seemed moderately sensitive to the apoptotic effects of TNF-α only at 72 h. MCF-7 M cells were sensitive to the antiproliferative effect of TNF-α but resistant to the cytotoxic effects of TNF-α. Examination of the TNFR expression revealed a similar expression of p75 TNFR2 among all three cell variants. p55 TNFR1 was expressed at the highest levels in MCF-7 N cells and MCF-7 L cells, whereas MCF-7 M cells expressed the lowest levels of TNFR1. The decreased expression of TNFR1 in MCF-7 M cells may account in part for their lowered sensitivity to TNF-α apoptosis as well as a lowered genera-

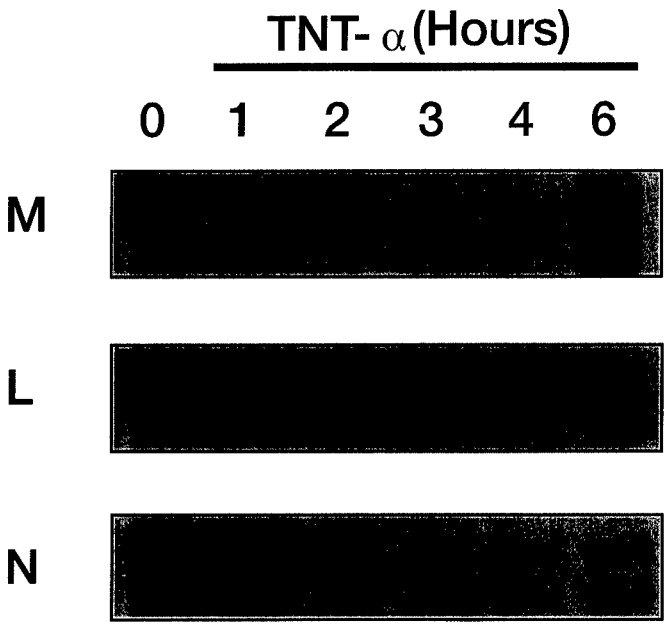

Fig. 7. Western blot analysis of PARP cleavage in MCF-7 cell variants. MCF-7 M, L, and N cells (2×10^6) were treated with TNF-α (10 ng/ml) for 1, 2, 3, 4, and 6 h. Cells were then harvested in PBS-EDTA and assayed for PARP cleavage as described in "Materials and Methods."

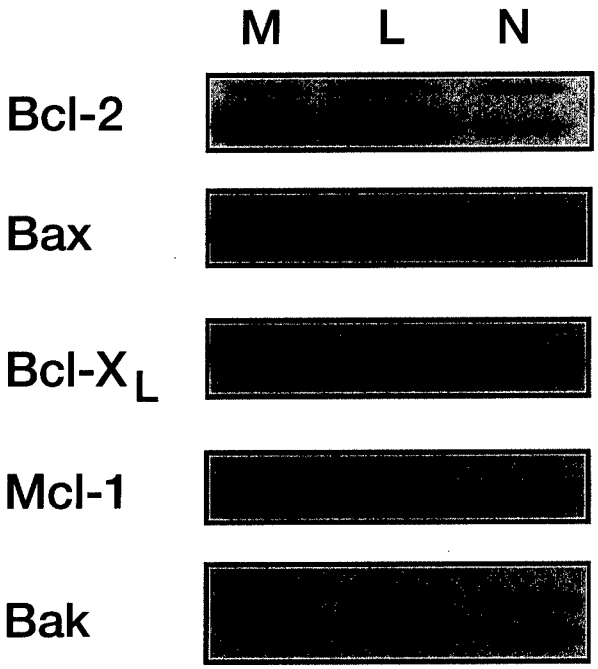

Fig. 8. Expression of Bcl-2 family proteins in MCF-7 cell variants. MCF-7 cell variants M, L, and N were grown for 2 days in normal media and harvested for Western blot analysis as described in "Materials and Methods" for the expression of Bcl-2, Bax, Bak, Bcl-X_L, and Mcl-1.

tion of ceramide. It is possible that despite lowered expression, TNFR1 or even TNFR2 may provide the antiproliferative signal in these cells. Given the role of ceramide in the inhibition of proliferation, the 1.7-fold increase in ceramide in the M cells may be sufficient for the suppression of cell proliferation but insufficient to induce apoptosis. We cannot rule out the possibility that altered expression or activation of TRADD, FADD, FLICE, or other proteins in the TNF signaling cascade may account for the inability to activate apoptosis in the M cells.

Examination of several members of the Bcl-2 family of apoptosis-regulating proteins suggests that the intrinsic resistance of the M cells and the delayed apoptotic DNA laddering and protease activation in the MCF-7 L cells as compared to the N variant were correlated with a higher expression of Bcl-2 and a lower expression of Bax. Many studies confirm that an increase in the expression of Bcl-2 correlates with resistance to apoptosis induced by a number of agents (34, 35). However, contradictory reports exist as to the ability of Bcl-2 or Bcl-X_L expression to inhibit TNF-α-induced apoptosis in MCF-7 cells. Vanhaesebroeck *et al.* (42) showed that overexpression of Bcl-2 in MCF-7 cells failed to offer a survival advantage to treatment with TNF-α. Conversely, Jaattela *et al.* (43) showed that overexpression of Bcl-2 and Bcl-X_L was correlated with an increased resistance to TNF-α-induced apoptosis. Again, these reported differences may be due to the individual MCF-7 cell variants used by each laboratory and may potentially be due to the variations in constitutive expression of other members of the Bcl-2 family, such as Bax. Overexpression of Bax or Bcl-X_S in MCF-7 cells resistant to chemotherapeutic drug treatment, serum starvation, and Fas-induced apoptosis has been shown to sensitize these cells to the induction of apoptosis (44–46). Thus, cells expressing high levels of Bax may not be as resistant to apoptosis, even when overexpressing Bcl-2. However, the Bcl-2 family of proteins may not account for all of the differences in apoptotic sensitivity reported here. Both the M and L stocks express similar levels of Bcl-2, Bax, Bak, Bcl-X, and Mcl-1; however, the L cells undergo apoptosis in response to TNF-α, whereas the M cells are resistant, suggesting that other differences within variants of MCF-7 cells will affect the antiapoptotic role of Bcl-2. Reports have indicated that Bcl-2 does not block ceramide generation but does inhibit ceramide analogue-induced apoptosis (47). Given the ability of Bcl-2 to block protease activation through the inhibition of cytochrome *c* release from the mitochondria (36, 37), the increased Bcl-2 expression and decreased Bax expression in MCF-7 L cells account for the delayed activation of PARP-specific caspases but not for the suppressed generation of ceramide.

Our results suggest a potential molecular basis for the differences in susceptibility to apoptosis among MCF-7 breast cancer cell variants. The increased generation of ceramide in the most apoptosis-sensitive variant cells (MCF-7 N) may account for their response to TNF-α as compared to the antiproliferative action of TNF-α in the less apoptosis-sensitive variants (MCF-7 M and L). This decreased ceramide generation may be due in part to decreased expression of TNFR1, as in the MCF-7 M cells, or possibly to an alteration in the ability of TNF-α to activate SMases, which may be the case in MCF-7 L cells. In MCF-7 cells, ceramide generation is early and transient, suggesting that its SMase activation is not a result of the apoptotic process but an early signaling intermediate. Gamen *et al.* (48) implicated CPP32/caspase 3 in the regulation of Fas-induced ceramide generation but not TNF-α-induced ceramide generation. Additionally, it was shown that REAPER-induced ceramide generation occurring at 1 h or later is blocked by an ICE-like protease inhibitor (49). We cannot rule out the possibility that ceramide generation may be mediated by events subsequent to FLICE/MACH1/caspase 8 or early caspase-dependent activation. In summary, our data indicate that the sensitivity of MCF-7 cells to apoptosis induced by TNF-α and other agents differs depending on the origin of the cells. Given the extensive use of MCF-7 cells as an ER-positive breast cancer model and a system for studying apoptotic signaling, the constitutive expression and regulation of apoptotic signaling molecules are therefore an important consideration.

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Effects of environmental estrogens on tumor necrosis factor α-mediated apoptosis in MCF-7 cells

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Environmental estrogens represent a class of compounds which have been shown to mimic the effects or activity of the naturally occurring ovarian hormone 17β-estradiol. Given the role of 17β-estradiol in cell survival in a number of systems, we wished to determine if environmental estrogens protect MCF-7 cells from apoptosis. Here we demonstrate that the organochlorine pesticides *o,p′* DDT and alachlor, like 17β-estradiol, have the ability to suppress tumor necrosis factor α (TNF)-induced apoptosis in estrogen receptor (ER)-positive MCF-7 breast carcinoma cells. These compounds, however, did not affect TNF-induced apoptosis of the ER-negative MDA-MB-231 cell line. The ability of these compounds to suppress apoptosis in MCF-7 cells was correlated with an ER-dependent increase in Bcl-2 expression. Taken together these results demonstrate that estrogenic organochlorine pesticides like *o,p′* DDT and alachlor may partially mimic the primary endogenous estrogen, 17β-estradiol, and function to suppress apoptosis in ER-responsive cells.

Introduction

Apoptosis is a process of cellular suicide by which specific cells undergo a programmed series of biochemical events culminating in the elimination of those cells (1,2). Apoptosis is a normal physiological process that functions to control cell populations during embryogenesis, immune responses, hormone withdrawal from dependent tissues and normal tissue homeostasis (1–6). Recent studies have suggested that apoptosis may play a critical role in the generation and progression of cancer and may have potential applications in cancer therapy (1,4–7).

Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissues. Both prostate and mammary epithelial cells undergo apoptosis upon removal of testosterone and estrogen, respectively (3,8–11). This dependence upon hormone for survival and proliferation extends to neoplasms arising from these tissues (3,9,12,13). The MCF-7 breast cancer cell line has been shown to form tumors in nude ovariectomized mice only in the presence of estrogen. Upon removal of estrogen the tumor cells begin to undergo apoptosis, leading to tumor regression (12). Additionally, recent studies have shown that pretreatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen (14–16). These reports also show that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis regulating proteins whose expression has been shown to suppress MCF-7 cell apoptosis (17). These studies provide evidence that estrogens may play a role in both tumorigenesis and drug resistance through suppression of apoptosis.

Environmental estrogens represent a class of compounds, both natural and synthetic, which can mimic the function or activity of the endogenous estrogen 17β-estradiol. These environmental estrogens may function as endocrine disrupters both in wildlife and humans, leading to developmental defects, disease and, potentially, cancer (18–23). Recently, a number of organochlorine pesticides, including DDT, (3α,5aβ,6β,9β,9aβ)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide (endosulfan II) and 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide (alachlor), have been shown to mimic estrogen and are capable of binding to the estrogen receptor (ER), causing transcription from estrogen response elements (ERE) in DNA and causing proliferation of MCF-7 cells *in vitro* (24–26). The potential exists that these compounds, acting through the ER, can affect the apoptotic pathways of estrogen-responsive cells. With mounting evidence for the role of estrogen in the regulation of apoptosis, we suggest that these environmental estrogens can act like endogenous estrogen to inhibit tumor necrosis factor α (TNF)-induced apoptosis in ER-positive breast cancer cells.

Abbreviations: alachlor, 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)-acetamide; *o,p′* DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethane; DMEM, Dulbecco's modified Eagle's medium; endosulfan II, (3α,5aβ,6β,9β,9aβ)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide: ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; TNF, tumor necrosis factor α.

Materials and methods

Cell culture

MCF-7 cells (N variant, passage 50) were generously provided by Louise Nutter (University of Minnesota) (27). MDA-MB-231 cells were obtained from The American Type Culture Collection (Rockville, MD). MCF-7 and MDA-MB-231 cells were routinely maintained and grown in high glucose-containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Gibco BRL, Gaithersburg MD) and 1×10^{-10} M porcine insulin (Sigma Chemical Co., St Louis, MO) (10% DMEM). Cells were maintained in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Prior to experiments both cells lines were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% DCC-FBS) supplemented with BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Gibco BRL) (5% CS-DMEM) for 48 h prior to plating. The cells were plated in 6-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. Following this, cells were treated accordingly.

17β-Estradiol was purchased from Amersham Corp. (Arlington Heights, IL), 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p'* DDT) was purchased from Sigma, alachlor and endosulfan II were purchased from AccuStandard (New Haven, CT).

Luciferase assays

Estrogen-responsive reporter gene analysis was performed as described by Klotz *et al.* (26). Briefly, MCF-7 cells were placed in 5% CS-DMEM for 48 h prior to plating. The cells were plated in 6-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 2 μg of plasmid pERE2-luciferase, which contains two copies of the vitellogenin ERE linked to the luciferase gene, and 1 μg of plasmid pCMV-β-galactosidase using 12 μl of lipofectamine (Gibco BRL). After 5 h, the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle, 17β-estradiol or environmental estrogen and incubated for 18 h at 37°C.

After 18 h the treatment-containing medium was removed and 250 μl of 1× lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) was added per well and incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15 000 *g* for 5 min. The cell extracts were normalized for protein concentration using the Bio-Rad Reagent following the supplied protocol (Bio-Rad, Hercules, CA). For β-galactosidase assays, the cell extract was placed in 500 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), 100 μl of *o*-nitrophenyl-β-D-galactopyranoside at 4 mg/ml in Z-buffer was added to each reaction and the tubes placed at 37°C. The addition of 400 μl of 1 M Na_2CO_3 terminated the reactions. The β-galactosidase activity of each reaction was measured at an absorbance of 420 nM. Luciferase activity for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Viability assay

Cell viability was determined using the Trypan blue assay as described previously (27). Briefly, MCF-7 or MDA-MB-231 cells were plated at 5.0×10^5 cells/ml in 10 cm^2 wells in 5% CS-DMEM. The cells were allowed to adhere for 24 h before treatment with vehicle (1% DMSO), 17β-estradiol (1 nM), *o,p'* DDT (100 nM) or alachlor (1 μM) for 24 h. Following this, cells were treated with TNF (10 ng/ml) (R&D systems, Minneapolis MN) and harvested 24 h later for viability analysis using the Trypan blue exclusion method. Percent viability was expressed as the percentage of viable cells in the treated samples as compared with control viability, with 500 cells counted per sample.

DNA fragmentation analysis

Following treatment, cells were harvested for DNA as described previously (27). Briefly, $1–2\times10^6$ cells were pelleted and resuspended in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 0.5% w/v SDS, pH 7.4) to which RNase A (100 μg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration 1 M) and lysates were incubated overnight at 4°C. Lysates were centrifuged at 15 000 *g* for 30 min and nucleic acids in the supernatant were precipitated in 2 vol of ethanol with 3 M Na acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Immunoblot analysis

MCF-7 cells (5×10^6) were grown for 3 days and then harvested in sonicating buffer (62.5 mM Tris–HCl, pH 6.8, 4% w/v SDS, 10% glycerol. 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, 25 mg/ml aprotinin) and sonicated for 30 s. Following centrifugation at 1000 *g* for 20 min, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline/0.05% Tween, 5% low fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with a solution of rabbit antisera (anti-Bcl-2 1:4000) and incubated for 2 h at room temperature. Blots were washed in phosphate-buffered saline/Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30 000 dilution; Oxford Scientific, Oxford, MI) for 30 min at room temperature. Following four washes with PBS/Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. After analysis, membranes were stained with Ponceau S to verify equal loading and transfer.

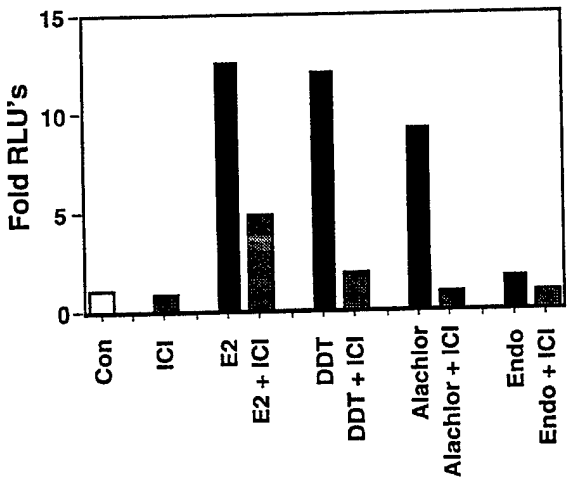

Fig. 1. Reporter gene activity of environmental estrogens. MCF-7 cells were transfected with 2 μg of ERE–luciferase containing reporter gene constructs. Cells were then treated with vehicle (control), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), 1 μM alachlor (Ala) or 1 μM endosulfan II (Endo) in the presence or absence of 100 nM ICI 182,780 (+ICI). Cells were harvested 18 h later for luciferase assays. Data is expressed as fold relative light units (RLU) over control from a representative experiment of three independent determinations.

Results

Several reports in the literature have addressed the estrogenicity of endocrine-disrupting chemicals in breast cancer cells. Klotz *et al.* showed that some organochlorine pesticides such as DDT metabolites and alachlor are capable of binding to the ER as measured by tritiated 17β-estradiol displacement (26). This group also showed that these compounds are capable of driving reporter gene transcription from ERE elements. These studies indicate the role of environmental estrogens in signaling through estrogenic pathways and that they, like 17β-estradiol, possess the ability to induce proliferation of MCF-7 cells (24–26). Based on these data we examined the estrogenic activity of estrogen and the environmental estrogens *o,p'* DDT and alachlor on MCF-7 cells. Using an estrogen-responsive reporter gene assay, 17β-estradiol (1 nM) treatment was shown to result in a 12.5-fold increase in luciferase activity (Figure 1). *o,p'* DDT (100 nM) resulted in an equivalent 12-fold increase in luciferase activity, while alachlor (1 μM) induced a 9.1-fold increase in luciferase activity. Addition of the pure anti-estrogen ICI 182,780 with either 17β-estradiol, *o,p'* DDT or alachlor reduced luciferase activity to 4.8-, 1.8-, 0.79- and 0.81-fold, respectively, indicating that the effect of these compounds on ERE-mediated transcriptional activity was dependent on the estrogen receptor. These results demonstrate that the two environmental estrogens *o,p'* DDT and alachlor, at doses 100 nM and 1 μM, respectively, activate ERE-mediated gene expression roughly equivalent to a 1 nM dose of the ovarian estrogen 17β-estradiol.

Based on the observation that estrogen is a survival factor in MCF-7 cells, we examined the ability of 17β-estradiol and the environmental estrogens *o,p'* DDT and alachlor to inhibit TNF-mediated cell death. We previously demonstrated that TNF strongly induced apoptosis in MCF-7 cells (27). Cell viability assay was used to assess the anti-apoptotic effects of 17β-estradiol and the environmental estrogens *o,p'* DDT and alachlor. TNF (10 ng/ml) caused a decrease in viability from 100% in the control to 33 ± 4.46% at 24 h. Pretreatment of

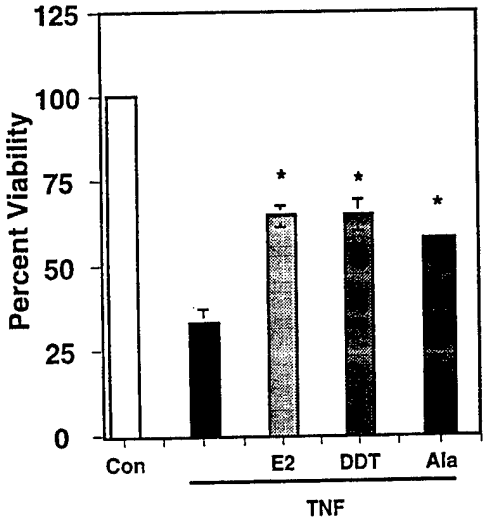

Fig. 2. Effects of estrogens on TNF-induced cell death in MCF-7 N cells. MCF-7 cells were treated with vehicle (Con), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cell were harvested 24 h later and viability was assessed using the Trypan blue method. Error bars represent standard deviation of the mean for five independent experiments performed in duplicate. Absence of error bars indicates <2.5% SD. Statistical significance (*) as compared with TNF treatment was determined using Student's *t*-test with $P < 0.02$ for Ala + TNF and $P < 0.005$ for 17β-estradiol + TNF and DDT + TNF.

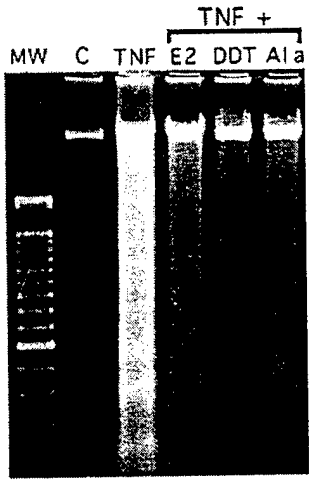

Fig. 3. Effects of estrogens on TNF-induced DNA fragmentation in MCF-7 N cells. MCF-7 cells were treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cells were harvested 48 h later for DNA fragmentation analysis. Molecular weight marker is shown as MW.

MCF-7 cells for 24 h with 1 nM 17β-estradiol resulted in a 31% inhibition of TNF-induced cell death, restoring viability to 64 ± 3.1% (Figure 2). Similarly 100 nM *o,p'* DDT reduced TNF cell death by 30.5%, restoring viability to 63.5 ± 4.46%. Treatment with 1 mM alachlor was able to inhibit cell death by 24%, restoring viability to 57 ± 1.33%. DNA fragmentation analysis was used to demonstrate that the suppression of TNF-induced loss of viability was due to induction of apoptosis (Figure 3). Consistent with previous results, TNF induced strong DNA fragmentation at 48 h (27). A 24 h pre-treatment with 17β-estradiol (1 nM), *o,p'* DDT (100 nm) or alachlor

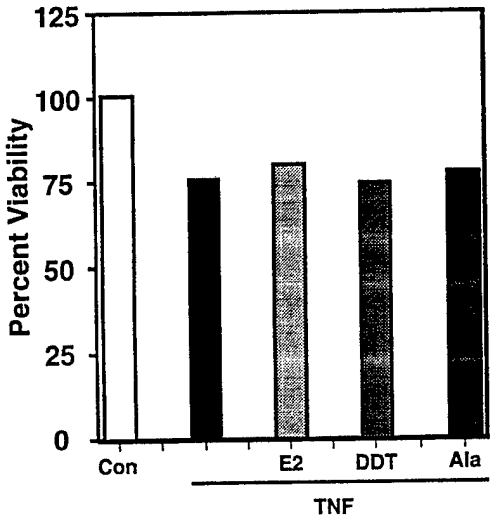

Fig. 4. Effects of estrogens on TNF-induced cell death in MDA-MB-231 cells. Cells were treated with vehicle (Con), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cells were harvested 24 h later and viability was assessed using the Trypan blue method. Data are expressed as percent viability from a representative experiment of two independent determinations.

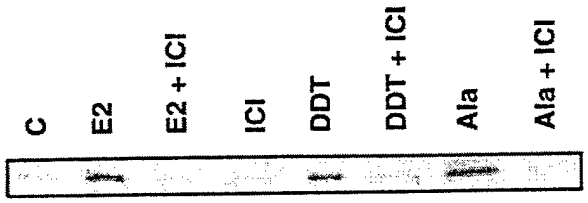

Fig. 5. Estrogen-induced Bcl-2 expression. MCF-7 cells were grown in 5% CS-DMEM medium for 5 days and subsequently treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT) or 1 μM alachlor (Ala) in the presence or absence of 100 nM ICI 182,780 (+ICI) as shown above. Cells were harvested 24 h later for western blot analysis of Bcl-2 expression.

(1 μM) all suppressed TNF-induced DNA fragmentation to near control levels.

To determine if the anti-apoptotic effects of these chemicals are exclusive to ER-positive breast cancer cells, we examined their effects on TNF-induced apoptosis in the ER-negative MDA-MB-231 breast cancer cell line. TNF (10 ng/ml) treatment induced a 25% decrease in MDA-MB-231 cell viability as compared with control cells at 24 h (Figure 4). Pretreatment of these cells with 17β-estradiol (1 nm), *o,p'* DDT (100 nM) or alachlor (1 μM) for 24 h prior to the addition of TNF (10 ng/ml) resulted in 21.2, 26.7 and 23.3% losses of viability, respectively, demonstrating that these compounds do not possess survival effects in ER-negative breast cancer cells.

Given that Bcl-2 is an estrogen-responsive gene, we examined the abilities of 17β-estradiol and the environmental estrogens *o,p'* DDT and alachlor to increase expression of this gene. MCF-7 cells were treated for 48 h with 17β-estradiol (1 nM), *o,p'* DDT (100 nM) or alachlor (1 μM) with or without pre-exposure to ICI 182,780 (100 nM). Western blot analysis revealed an increase in expression of Bcl-2 with all three compounds which was inhibited by ICI 182,780 treatment, indicative of a specific ER-mediated pathway (Figure 5).

Discussion

Environmental estrogens represent a class of compounds which possess the ability to mimic the activity and effects of endogenous 17β-estradiol. In addition to their role as endocrine disrupters in wildlife, these compounds may also affect humans, resulting in developmental defects, disease and, potentially, cancer. 17β-Estradiol induces proliferation of the ER-positive MCF-7 breast cancer cell line and also acts as a survival factor in these cells in response to treatment with the anti-estrogen tamoxifen, as well as with chemotherapeutic drugs (14–16). The ability of environmental estrogens to mimic 17β-estradiol and cause proliferative and estrogenic effects has been previously analyzed in MCF-7 breast cancer cells (24–26). Given the previously described estrogenicity of these compounds and the role of 17β-estradiol in cell survival, we examined the effects of two organochlorine pesticides on suppression of apoptosis in human breast cancer cells.

We have previously demonstrated that TNF acts as a potent inducer of apoptosis in sensitive MCF-7 cells (27). Consistent with the previous demonstration of the survival effect of estrogen, we show that 1 nM 17β-estradiol is capable of partially suppressing TNF-induced apoptosis in MCF-7 cells. The abilities of both *o,p'* DDT and alachlor to protect against TNF-induced cell death in MCF-7 cells closely correlate with the relative estrogenic potential of these compounds. The most estrogenic compounds in the reporter gene assay, 17β-estradiol (at 1 nM) and *o,p'* DDT (at 100 nM), exerted the greatest survival effects against TNF-induced cell death. While alachlor, a less potent ER agonist even at 1 μM, exerted the least effect on suppression of TNF cytotoxicity. However, upon analysis of DNA fragmentation induced by TNF we observed a significant reduction in apoptosis by pretreatment with all three agents, suggesting that subtle differences in viability may not directly correlate with qualitative analysis of apoptosis.

Recently, Shen *et al.* demonstrated that an isomer of DDT, *p,p'* DDT, was capable of activating cellular signaling events in ER-negative MCF-10A cells, suggesting that some organochlorine pesticides or potentially environmental estrogens may function through other signaling pathways (28). To investigate the possibility that either alachlor or *o,p'* DDT affects apoptosis in an ER-independent manner, we used the ER-negative MDA-MB-231 cell line. ER-negative MDA-MB-231 breast cancer cells were not as sensitive to the cytotoxic effects of TNF as MCF-7 cells. As expected, in ER-negative MDA-MB 231 cells 17β-estradiol exerted no protective effect against TNF-induced cell death. Similar to the effects of 17β-estradiol, pretreatment of these cells with either *o,p'* DDT or alachlor did not affect the ability of TNF to induce cell death, suggesting that at the concentrations used and under the conditions tested here the effects observed on apoptosis are occurring through an ER-dependent pathway. However, we cannot rule out the possibility that these compounds may possess anti-apoptotic effects not observed here which occur through an ER-independent mechanism.

Recent reports show that one mechanism by which estrogens may affect apoptosis is through increased expression of Bcl-2, a member of a family of apoptosis regulating proteins (14–16). Additionally, Jaattela *et al.* have demonstrated that overexpression of Bcl-2 in MCF-7 cells resulted in resistance to TNF-induced apoptosis (17). The data presented here show that, like estrogen, the two organochlorine compounds *o,p'* DDT and alachlor both increase Bcl-2 expression in an ER-dependent manner, suggesting that the mechanism of organochlorine suppression of apoptosis is in part mediated through increased expression of Bcl-2.

We have provided evidence that the organochlorine compound *o,p'* DDT, which can function as an environmental estrogen, is capable of suppressing TNF-induced apoptosis in human breast cancer cell lines and this effect is correlated with increased expression of Bcl-2. We have also demonstrated that the anti-apoptotic effect of this compound is observed in ER-positive MCF-7 cells but not in ER-negative MDA-MB-231 cells, suggesting specificity of this effect for the ER-mediated pathway. Environmental estrogenic compounds which mimic estrogen have been shown to induce proliferative responses and expression of estrogen-responsive genes and promoters. Here we demonstrate that certain environmental estrogens are also capable of mimicking estrogen in their ability to suppress apoptosis.

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Differences in protein kinase C and estrogen receptor α, ß expression and signaling correlate with apoptotic sensitivity of MCF-7 breast cancer cell variants

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Abstract. Widespread use of MCF-7 human breast cancer cells as a model system for breast cancer has lead to variations in these cells between different laboratories. Although several reports have addressed these differences in terms of proliferation and estrogenic response, differences in sensitivity to apoptosis have just begun to be described. Based on the possible differences in apoptotic sensitivity that may arise due to the existence of MCF-7 cell variants, we determined the relative sensitivity of MCF-7 cell variants from three established laboratories (designated M, L and N) to known inducers of apoptosis. Consistent with our previous studies we demonstrate that differences exist among these variants in regards to tumor necrosis factor α (TNF)-induced cell death and inhibition of proliferation in a dose-dependent manner. To establish if the difference in apoptotic susceptibility was specific to TNF, the three MCF-7 cell variants were tested for their response to other known inducers of apoptosis: okadaic acid, staurosporine and 4-hydroxy-tamoxifen. Viability and DNA fragmentation analysis revealed a similar pattern of resistance to apoptosis by all agents in the MCF-7 M variant. The MCF-7 L variant was resistant to okadaic acid and 4-hydroxy-tamoxifen but not staurosporine. In contrast, MCF-7 N cells were sensitive to induction of apoptosis by all agents. The role of both protein kinase C (PKC) and estrogen signaling in the regulation of cell survival prompted investigation of these pathways as a mechanism for differential sensitivity of MCF-7 cell variants to apoptosis. While both estrogen receptor α (ERα) and ERß were expressed in MCF-7 M and N cells, the absence of ERß in MCF-7 L cells correlated with decreased estrogen responsiveness of the L variant. Variations in estrogenic responsiveness and PKC isoform expression may account for the enhanced susceptibility of both the L and N variants to staurosporine.

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Abbreviations: TNF, tumor necrosis factor α; PgR, progesterone receptor; ER, estrogen receptor; ERα, estrogen receptor α; ERß, estrogen receptor ß; E2, 17ß-estradiol; OHT, 4-hydroxy-tamoxifen; PKC, protein kinase C; PMA, phorbol ester; IC_{50}, inhibitory concentration 50%

Key words: MCF-7 cells, apoptosis, protein kinase C, estrogen receptor, tumor necrosis factor

Introduction

Since the establishment of the MCF-7 cell line in 1973 from a woman with metastatic breast cancer, these cells have been extensively used world wide as a model for ER positive breast cancer (1,2). This widespread use of MCF-7 cells has lead to the generation of MCF-7 cell variants, which represent phenotypic alterations within the cell line that arise among separate stocks of MCF-7 cells maintained in different laboratories. Previous studies have suggested that various MCF-7 cell line variants may possess intrinsic variations in estrogen responsiveness and proliferation rates. Osbourne *et al* reported that MCF-7 cells obtained from different laboratories exhibited differences in proliferation, estrogen receptor and progesterone receptor (PgR) levels, estrogen and anti-estrogen responses, and tumorigenicity (3). Klotz *et al* showed that different variants of MCF-7 cells displayed different levels of variant ER mRNAs which were correlated with their differential response to estrogen stimulation (4). MCF-7 variants tested by Villalobos *et al* exhibited differing responses to 17ß-estradiol- and environmental estrogen-induced proliferation and expression of the estrogen responsive genes pS2 and cathepsin-D (5). The reported differences in MCF-7 cell variants lends support to the notion that cellular context may predict susceptibility to apoptosis.

The differences in MCF-7 cell variants are hypothesized to extend to apoptosis. A wide variety of agents have been shown to induce apoptosis in MCF-7 cells including okadaic acid, staurosporine, TNF, Fas, retinoic acid, vitamin D analogues, 4-hydroxy-tamoxifen, ceramide analogues, hormone withdrawal and various chemotherapeutic drugs (6-20). With respect to TNF, contradictory reports have been published regarding the sensitivity to induction of apoptosis and inhibition of proliferation (17-19). We have recently demonstrated that differences in susceptibility to TNF-induced apoptosis exist among MCF-7 cell variants suggesting that discrepancies in the literature may be due to differences in the MCF-7 cell variant used (21).

Our previous study concluded that differences in sensitivity among MCF-7 cell variants might be partially due to altered regulation of critical apoptotic signaling events. Expression of members of the Bcl-2 family of proteins has been shown to regulate apoptotic signaling decisions, and these proteins represent a number of related proteins whose expression has been shown to regulate apoptosis (22,23). This family is comprised of both anti-apoptotic and pro-apoptotic members, the relative expression of which in part determines apoptotic sensitivity and activation of executioner caspases. Our previous results also demonstrated that apoptotic differences in MCF-7 cells correlated with differential expression or lack of expression of various pro- and anti-apoptotic members of the Bcl-2 family, Bax and Bcl-2, respectively (21).

Steroid hormones also regulate apoptosis in hormone-responsive tissues. This dependence upon hormones for survival and proliferation extends to neoplasms arising from these tissues (15,24,25). Recent studies have shown that pre-treatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen (14,26-29). These reports also demonstrate that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, whose expression has been shown to suppress apoptosis of MCF-7 cells (14, 26-28,30). Therefore the estrogen receptor (ER) pathways may influence cell survival decisions through the expression of Bcl-2 expression or potentially other cell survival proteins. The recent identification of a second estrogen receptor ß (ERß) suggests another pathway through which estrogenic signals might regulate biologic effects (31-34). Several reports have demonstrated the expression of ERß in MCF-7 cells (35-37), while others observed very low or absent expression of ERß (38-40). These published differences of ERß expression, like the observed differences in apoptosis, may be due to the MCF-7 cell variant used. Given the involvement of the 17ß-estradiol-ER in suppression of apoptosis, the expression of ERß may also function in the regulation of estrogen's survival effects and differences in the apoptotic sensitivity of the MCF-7 cell variant used.

A number of studies have implicated PKC in the regulation of apoptosis in a cell type and isoform-specific fashion. The members of the PKC family are divided into three sub-groups based upon their mechanism of activation. The classical isoforms (α, ßI, ßII, γ) are activated by both calcium and diacylglycerol; the non-classical isoforms (δ, ε) are activated by diacylglycerol. While the atypical (ζ, λ/ι) are activated in a calcium and diacylglycerol independent manner (41,42) PKC-α has been associated with the regulation of multi-drug resistance in phenotype in MCF-7 cells (43,44) as well as a more aggressive phenotype as evidenced by increased proliferation rate (45). However, Manni *et al* demonstrated that overexpression of PKC-α in their MCF-7 cells induced a more differentiated phenotype with decreased proliferative rate (46). The effects of specific PKC isoform expression may therefore be dependent on the cellular context of the MCF-7 cell variant used. These differences extend to the responsiveness of MCF-7 cells to PKC activating phorbol esters (PMA). Phorbol esters induce apoptosis in MCF-7 cells with overexpression of PKC-α indicating that alterations in the PKC pathway can modulate the decision of breast cancer cells to undergo death or differentiation (47). PMA is also capable of increasing multi-drug resistance as well as suppression of the induction of apoptosis in MCF-7 cells [(48,49), unpublished data]. Because of the role of PKC in regulating a diverse number of cellular processes, it is both the activation of and relative expression of various PKC isoforms that may determine the cellular response to apoptotic stimuli.

Here we further investigated differences in MCF-7 cell variant susceptibility to cell death induced by several known apoptotic inducers. We demonstrate that the relative sensitivity of these three cell variants may be in part due to differences in the constitutive expression of PKC isoforms as well as differences in both the estrogen response and expression of ERα and ERß.

Materials and methods

Cell culture and reagents. MCF-7 cell variants M and L were a gift from Dr Stephen M. Hill (Tulane University). The MCF-7 M cell variant (passage 180) originated from the laboratory of the late Dr William McGuire (University of Texas). The MCF-7 L cell variant (passage 40) originated from the laboratory of Dr Marc Lippman (Georgetown University). MCF-7 N cell variant (passage 50) is a subclone of MCF-7 cells from American Type Culture Collection (Rockville, MD) generously provided by Dr Louise Nutter (University of Minnesota). MDA-MB-231 (p170) and MCF-7ADR (p10) cells were obtained from the Lombardi Cancer Center Tissue Culture Core Facility of Georgetown University Medical Center. All cells were routinely maintained and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Gibco BRL, Gaitherburg, MD) and porcine insulin $1x10^{-10}$ M (Sigma Chemical Co., St. Louis, MO) (10% DMEM). For some studies media was changed to serum and insulin-free media DMEM supplemented with BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin and sodium pyruvate (0% DMEM).

ICI 182,780 {7α-[9-(4,4,5,5,5-pentafluoropentasulfinily) nonyl]estra-1,3,5(10)triene-3,17ß-diol} was generously provided by Dr Alan E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). 4-hydroxy-tamoxifen was obtained from Sigma. TNFα, was obtained from R&D systems. Staurosporine and sodium okadaic acid were obtained from LC laboratories. C6-ceramide and paclitaxel were obtained from BioMol. Doxorubicin and was obtained from ICN.

Viability assays. MCF-7 cells from variants M, L and N were plated at $5.0x10^4$ cells/ml in 10 cm^2 wells. The cells were allowed to adhere for 18 h before treatment with staurosporine, sodium okadaic acid, 4-hydroxy-tamoxifen. Following treatment for 24 h cells were harvested in PBS with EDTA (1 mM) and counted for viability. The results are represented as the number of apoptotic cells as measured by trypan blue exclusion. Apoptosis was expressed as the percentage of cells stained with trypan blue in treated samples from control viability.

The crystal violet viability assay was performed as previously described by Cai *et al* (18). Briefly, $1x10^4$ cells/well of each variant (M, L and N) were plated into a 96-well plate in 10% DMEM. The media was removed the following day and replaced with 0% DMEM and the cells were treated with the appropriate concentration of doxorubicin (0.01-10.0 µg/ml) or paclitaxel (0.0005-5.0 µg/ml) for times indicated. Following treatment the media was aspirated from each well and cells were stained with a (0.5%) solution of crystal violet for 10 min. The crystal violet was removed, cells were washed twice with PBS followed by lysis in a 0.1% SDS solution. Absorbancy at 595 nm was measured using an ELX 808 BioTek microplate reader. Percent viability was determined as compared to control samples and IC_{50} values were determined based upon this data. Data are representative of 3 independent experiments performed each with 24 replicates.

The CellTiter 96™ AQueous viability/proliferation assay (Promega) is a modified tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium), inner salt; MTS] assay performed according to manufacturers specifications. Briefly, $1x10^4$ MCF-7 cells were plated into 96-well plates with 10% DMEM overnight. The following day the media was replaced with fresh 10% DMEM and cells were treated with or without TNFα at the doses indicated. Following treatment for the times indicated the media was removed and the MTS assay reagents was added to the cells for 30 min and the absorbancy at 490 nM was determined using an ELX 808 BioTek microplate reader. Data are representative of the normalized absorbance (relative viability) of 3 independent experiments with 4 replicates each.

DNA fragmentation analysis. Following treatment, cells were harvested for DNA as described previously (21). Briefly, $1-2x10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS (w/v) pH 7.4] to which RNase A (100 µg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration, 1 M) and lysates were incubated overnight at 4°C. Lysates were centrifuged at 15,000 x g for 30 min, and nucleic acids in the supernatant were precipitated in two volumes of ethanol with 50 mM Na acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Luciferase assays. Estrogen responsive reporter gene analysis was performed as described by Klotz *et al* (50). Briefly, MCF-7 cells were placed in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% dextran-coated charcoal-treated FBS (5% DCC-FBS) for 48 h prior to plating. The cells were plated in 6-well plates at $5x10^5$ cells/well in the same media and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 2 µg of pERE2-luciferase plasmid, which contains two copies of the vitellogenin ERE linked to the luciferase gene, along with 1 µg of the pCMV-ß-galactosidase plasmid using 12 µl of Lipofectamine (Gibco BRL, Gaitherburg, MD). After 5 h, the transfection media was removed and replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle or 17ß-estradiol (1 nM) and incubated for 18-24 h at 37°C.

After treatment media was removed and 250 µl of 1X lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) was added per well and incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15,000 g for 5 min. The cell extracts were normalized for protein concentration using the Bio-Rad reagent following the supplied protocol (Bio-Rad Laboratories, Hercules, CA). For ß-galactosidase assays, the cell extract was placed in 500 µl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM ß-mercaptoethanol), 100 µl of o-nitrophenyl-ß-D-galactopyranoside at 4 mg/ml in Z-buffer added to each reaction and the tubes placed at 37°C. The addition of 400 µl of 1 M Na_2CO_3 terminated the reactions. The ß-galactosidase activity of each reaction was measured at an absorbance of 420 nM. Luciferase activity for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

RT-PCR analysis of ERα and ERß expression. RNA was extracted using Ultraspec™ RNA isolation kit (Biotecx Lab). In brief, frozen cell pellets ($1x10^6$ cells) were lysed in 1 ml Ultraspec™ RNA solution, Then 0.2 ml chloroform was added to the cell lysate and shaken. After centrifugation, the colorless upper aqueous phase were removed to a new tube. An equal volume of isopropanol was added and the RNA was precipitated by centrifugation. Then, the RNA was washed with 75% ethanol and recovered in DEPC-treated water. RT-PCR were taken using a Perkin-Elmer RNA PCR kit following manufacturer's protocol. Primers were synthesized from Life Technologies. Primers used to amplify the ERα were: 5'-TGC CAA GGA GAC TCG CTA-3' (nucleotides 894-912) and 5'-TCA ACA TTC TCC CTC CTC-3' (nucleotides 1139-1157), giving an amplified product of 263 bp (51). For ERß, primer sequences were: 5'-TTC CCA GCA ATG TCA CTA ACT-3' (nucleotides 33-53) and 5'-TCT CTG TCT CCG CAC AAG G-3' (nucleotides 539-558), giving an amplified product of 525 bp (52). To check cDNA integrity, fragments of glyceraldehyde phosphate-3-dehydrogenase (GAPDH) were also amplified in parallel. The primers for GAPDH were: 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3', giving a PCR product of 452 bp.

Western blot analysis. Western blot analysis was performed as described previously (53). MCF-7 cells were grown for two days as described above and then $5x10^6$ cells were harvested

in sonicating buffer [62.5 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol]. 1 mM phenylmethylsulfonyl fluoride (PMSF), (25 μg/ml leupeptin, 25 μg/ml aprotinin) and sonicated for 30 sec. Following centrifugation at 1,000 x g for 20 min, 50 μg of protein was resuspended in sample loading buffer [62.5 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% ß-mercaptoethanol, 0.01% bromophenol blue], boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS-Tween (0.05%) - 5% lowfat dry milk solution at 4°C overnight. The membrane was subsequently incubated with monoclonal antibodies specific to protein kinase C isoforms (Oxford, MI) or with a monoclonal antibody specific to ERα [1:5,000, hERa-Ab8 (clone AER 311), NeoMarkers Inc. (Freemont, CA)], for 2 h at room temperature. Blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat anti-mouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Oxford, Oxford, MI) for 30 min at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad) and fold differences in expression between the three cell variants are representative of densitometric differences as compared to background ± standard deviation from two independent experiments.

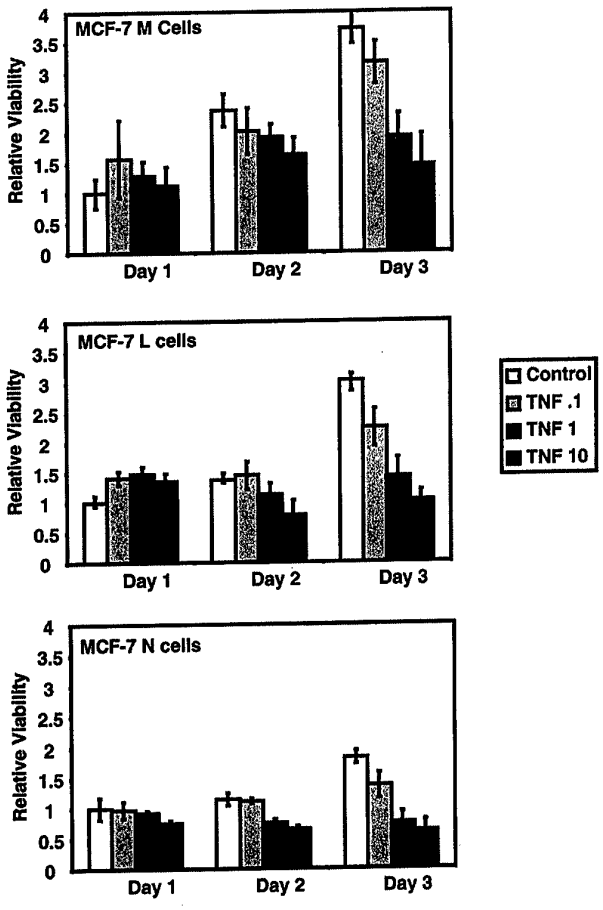

Figure 1. Dose-dependent cytotoxic/cytostatic effects of TNF in MCF-7 cell variants. MCF-7 variants M, L, or N were treated with or without TNF (0.1-10 ng/ml) for 1, 2 or 3 days. Relative viability was determined from normalized absorbancy as compared to day 1 control (1.0). Error bars represent standard error for three experiments with four replicates each.

Results

Previously, our laboratory reported that three MCF-7 cell variants referred to as M, L and N displayed differences in susceptibility to TNF-induced apoptosis (21). Using a viability/proliferation assay over three days we further explored the dose-dependent effects of TNF on these cell variants (Fig. 1). Treatment of MCF-7 M and L variants with TNF (0.1-10 ng/ml) resulted in decreased proliferation from control levels at both days 2 and 3. Interestingly, in both M and L cell variants TNF (0.1-10 ng/ml) increased relative viability at day 1 which is suggestive of a potentially early enhancement of proliferation. The relative viability of both M and L cell variants treated with TNF (0.1-10 ng/ml) remained at or above the day 1 control value with TNF (10 ng/ml) treatment of the L variant slightly decreasing relative viability 22% below 48 h control values. The MCF-7 N variant displayed a dose-dependent loss of viability as early as day 1 which was decreased below the 24-h control relative viability values for both 1 and 10 ng/ml over 3 days. TNF treatment of N cells with 0.1 ng/ml of TNF appeared to inhibit proliferation suppressing relative viability at or slightly above the day 1 control values. This demonstrates that among these three MCF-7 cell variants there is an altered sensitivity to TNF-induced cell death and inhibition of proliferation with most to least resistant being M > L > N. To determine if resistance to TNF-induced apoptosis in the L and M variants was specific only to TNF or due to a more universal mechanism of apoptotic resistance the three MCF-7 variants were analyzed for their response to other known inducers of apoptosis. The broad spectrum kinase inhibitor staurosporine, the phosphatase inhibitor okadaic acid and the anti-estrogen 4-hydroxy-tamoxifen have been previously shown to induce apoptosis in MCF-7 cells (6,12,14,20). Staurosporine (1 μM), okadaic acid (100 nM) and 4-hydroxy-tamoxifen (10 μM) were tested for their ability to induce apoptosis in the three cell variants as measured by trypan blue viability and DNA ladder formation at 48 h. Both the M and L variants were resistant (<10% loss of viability) while the N variant was sensitive (39±3.0% and 70±22% loss of viability) to apoptosis induced by okadaic acid and 4-hydroxy-tamoxifen respectively (Fig. 2). The loss of viability in the N variant was due to apoptosis as confirmed by DNA fragmentation analysis. As expected no fragmentation was observed in either the M or L variants with either okadaic acid or 4-hydroxy-tamoxifen (Fig. 3). It was also determined that only the N variant underwent apoptosis in response to C6-ceramide treatment as measured by DNA ladder formation and loss of viability (data not shown). Treatment of M, L and N cell variants with staurosporine resulted in a decrease in

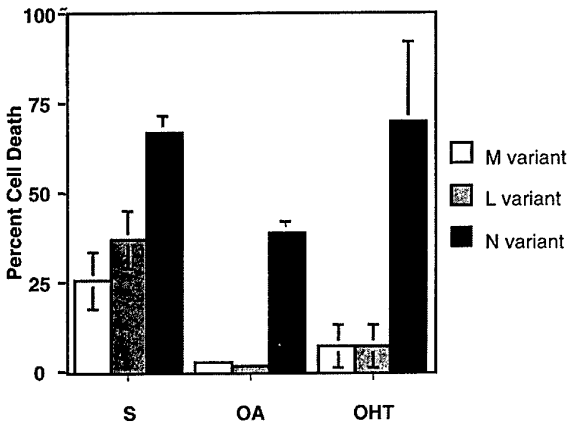

Figure 2. Effects of apoptotic inducers on cell viability in MCF-7 cell variants. Percent cell death as measured by trypan blue staining at day 2 of MCF-7 cell variants M, L and N treated with either staurosporine (S) (1 μM), okadaic acid (OA) (100 nM) or with 4-hydroxy-tamoxifen (OHT) (10 μM). Error bars represent standard deviation for three experiments performed in duplicate. Absence of error bars represents less than 3% error between replicates.

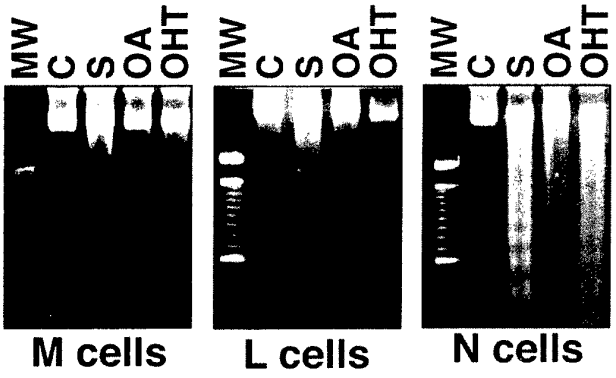

Figure 3. DNA fragmentation in MCF-7 cell variants by apoptotic inducers. Analysis of DNA fragmentation of MCF-7 cell variants M, L and N by agarose gel electrophoresis. Cells were treated with either 0.1% DMSO (C), staurosporine (S) (1 μM), okadaic acid (OA) (100 nM) or with 4-hydroxy-tamoxifen (OHT) (10 μM) and harvested for DNA fragmentation analysis after 48 h exposure. MW represents a molecular weight standard run with each variant.

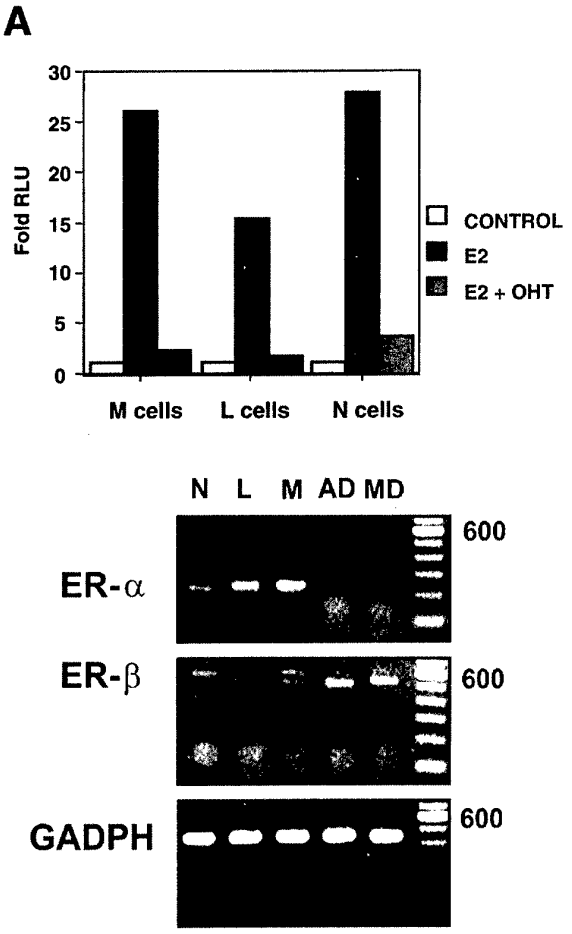

Figure 4. Estrogen-mediated gene transactivation in MCF-7 M, L and N variants. A, MCF-7 cell variants ($1x10^6$) were transfected with 2 μg of ERE-luciferase reported plasmid. Following this, MCF-7 cells were treated with vehicle (control), 1 nM 17ß-estradiol alone (E2) or with 17ß-estradiol and 100 nM 4-hydroxy-tamoxifen (E2 + OHT). Cells were harvested 18 h later for luciferase assay. Data is expressed as fold relative light units (RLUs) over control from a representative experiment of 3 individual experiments. B, MCF-7 cell M, L and N variants, MDA-MB-231 or MCF-7ADR cells were analyzed by RT-PCR for expression of ERα or ERß.

viability of 25±2.5%, 37±8.0% and 65±4.5%, respectively. DNA ladder analysis revealed that the decrease in viability observed in the L and N variants resulted from an induction of apoptosis. Despite the moderate loss of viability with staurosporine the M variant did not undergo observable DNA fragmentation. The chemotherapeutic drugs, doxorubicin and paclitaxel, have both been previously described to induce apoptosis in MCF-7 cells (16,26). We examined the ability of both doxorubicin over a dose range of 0.01-10 μg/ml and taxol over a dose range of 0.0005-5.0 μg/ml to induce cell death in MCF-7 cell variants. Using a crystal violet viability assay we determined the IC_{50} values for both doxorubicin and paclitaxel in all three cell variants. After 48 h of treatment with doxorubicin IC_{50}s for the three variants were in order of decreasing resistance: M (4.93 μg/ml) > L (1.72 μg/ml) > N (0.113 μg/ml). A similar trend was observed after 48 h of treatment with paclitaxel, with IC_{50}s in order of decreasing resistance being: M (1.732 μg/ml) > L (0.68 μg/ml) > N (0.046 μg/ml). These results demonstrate a consistent increased resistance of both MCF-7 M and L variants to both paclitaxel and doxorubicin.

Estrogenic responses of the three variants were analyzed to determine if differences in variant response might account for the above differences in apoptotic sensitivity. Analysis of ERE-linked reporter gene expression revealed that in the presence of 17ß-estradiol (1 nM), variants M, L and N expressed 26-, 15.4- and 28-fold increases in luciferase activity over control, respectively (Fig. 4A). These differences in estrogen signaling between the three variants prompted investigation into ERα and ERß expression. All three variants expressed ERα as measured by RT-PCR analysis (Fig. 4B). Lack of expression of ERα was demonstrated in the ER negative MDA-MB-231 and MCF-7ADR cell lines. Similar levels of ERα protein between the three variants were

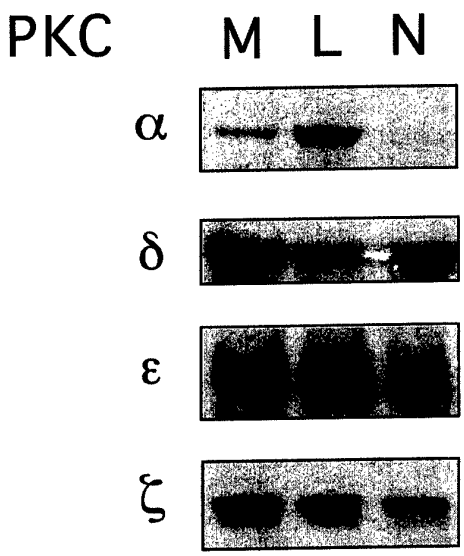

Figure 5. Expression of PKC isoforms in MCF-7 cell variants. MCF-7 cell variants M, L and N were grown for 2 days in normal media and harvested for Western blot analysis as described in Materials and methods for expression of PKC isoforms α, δ, ε, ζ.

Table I. Quantitation of PKC isoform expression in MCF-7 cell variants.[a]

PKC isoform	MCF-7 M	MCF-7 L	MCF-7 N
PKC-α	1.99±0.43	3.34±0.27	1.46±0.08
PKC-δ	2.75±0.28	1.96±0.29	2.83±0.12
PKC-ε	3.83±0.54	3.66±0.29	3.57±0.51
PKC-ζ	2.25±0.34	1.93±0.02	1.71±0.02

[a]MCF-7 cell variants M, L and N were analyzed for expression of PKC isoforms (α, δ, ε, ζ) by Western blot. Expression was quantitiated using imaging densitometry of two independent experiments ± SD with data represented as fold expression from normalized background density of 1.

confirmed by Western blot analysis (data not shown). Consistent with previous reports (36,37,40), MDA-MB-231 cells strongly expressed ERß as measured by RT-PCR analysis (Fig. 4B). Interestingly, MCF-7ADR cells, which are purportedly ER negative (54,55), displayed similar expression of ERß as compared to the MDA-MB-231 cells. Several previous reports have suggested that MCF-7 cells either lack or weakly express ERß as compared to other cell lines such as MDA-MB-231 (35-40). Our results demonstrate distinct differences in ERß expression among the three MCF-7 cell variants. While MCF-7 L cells display no ERß expression both MCF-7 N and M cells demonstrate weak ERß expression. Several reports have also demonstrated the existence of ERß RNA variants expressed distinctly in different cell types. The variants may function in different cell types as dominant negative or to enhance estrogen response (37,40,56). As apparent from the RT-PCR analysis of ERß expression some variant mRNAs may be present in both MDA-MB-231 and MCF-7 M cells.

At the concentrations used to induce apoptosis, staurosporine has been shown to act as a broad-spectrum protein kinase inhibitor, including inhibition of PKC as well as tyrosine kinases (57). The differences in sensitivity between the M, L and N variants to apoptosis or estrogen response may potentially be influenced by differences in PKC isoform expression. Western blot and subsequent densitometric analysis revealed the greatest expression of PKC-α in MCF-L > M > N cells with 56 and 40% greater expression than M or N respectively (Fig. 5, Table I). Expression of PKC-δ was least in MCF-7 L cells, with MCF-7 M and N cells expressing a nearly equivalent 29 and 31% greater respective expression than the L variant. Very similar levels of expression of PKC-ε were observed among the three variants, with the M variant possessing 4 and 7% greater expression than L and N respectively. Expression of PKC-ζ was also highest in MCF-7 M cells with a 15 and 24% higher expression than L and N respectively. MCF-7 cell variants were also analyzed for the expression of PKC-ßI and PKC-γ, neither of which was observed, as well as PKC-ι, levels of which did not differ between any of the three cell variants.

Discussion

Reported discrepancies exist concerning the apoptotic responses of MCF-7 cells to anti-Fas antibody and TNF. Several studies have indicated that MCF-7 cells are readily induced to undergo apoptosis in response to TNF and anti-Fas (7,8). However, some reports have indicated that TNF and Fas only weakly induce apoptosis in MCF-7 cells (19,21,58,59). Others have shown that the cytotoxic versus the cytostatic effects of TNF depend on the media and serum conditions used to culture the MCF-7 cells (60). Our previous results indicate that the apoptotic response of MCF-7 cells may be due to variations among different variants of these cells and dependence on the constitutive expression of apoptotic regulating proteins or alteration in apoptotic signaling within these cell variants which determines their relative sensitivity. Further investigation into the response to TNF among these three variants revealed interesting dose-dependent effects. Our results suggests that in the M and L cell variants TNF treatment resulted in a dose-dependent suppression of proliferation over three days with slight cytotoxicity only observed in the L variant at the highest concentration of TNF (10 ng/ml). The anti-proliferative effects of TNF in M and L variants along with the cytotoxic effects in the N variant are consistent with our previous results (21) and are suggestive of an altered dose response among the three variants. The observations of the differences in susceptibility to TNF's effects prompted us to examine the ability of other known cytotoxic agents to induce apoptosis in these cell variants. We show that while the MCF-7 N variant was sensitive to apoptotic induction by okadaic acid, 4-hydroxy-tamoxifen and C6-ceramide, both the M and L cell variants were resistant. Staurosporine, however, did induce apoptosis in both the MCF-7 L and N variants. Our

results demonstrate that the three MCF-7 cell variants display differential sensitivity to apoptosis with the M variant being resistant to all agents tested, the L variant displaying sensitivity only to staurosporine. The MCF-7 N variant was sensitive to all inducers of apoptosis tested; TNFα, staurosporine, okadaic acid, C6-ceramide and 4-hydroxy-tamoxifen. Taken together, these data suggest that the greater resistance among the M and L variants may be due to a generalized mechanism of resistance absent in the N variant.

Many studies confirm that an increase in expression of Bcl-2 correlates with resistance to apoptosis induced by a number of agents (22,23). Contradictory reports however exist as to the ability of Bcl-2 or Bcl-X_L expression to inhibit TNF-induced apoptosis in MCF-7 cells. Vanhaesebroeck *et al* showed that overexpression of Bcl-2 in MCF-7 cells failed to offer a survival advantage to treatment with TNF (61). Conversely, Jaattella *et al* showed that overexpression of Bcl-2 and Bcl-X_L was correlated with an increased resistance to TNF apoptosis (62). Again these reported differences may be due to the individual MCF-7 cell variant used by each laboratory and potentially the variations in constitutive expression of other members of the Bcl-2 family such as Bax. Overexpression of Bax or Bcl-X_S in MCF-7 cells resistant to chemotherapeutic treatment, serum starvation and Fas-induced apoptosis has been shown to sensitize these cells to induction of apoptosis (63-65). Thus, cells expressing high levels of Bax may not be as resistant to apoptosis even when overexpressing Bcl-2. The Bcl-2 family of proteins however, may not account for all of the differences in apoptotic sensitivity reported here. We have previously shown that both the M and L variants express similar levels of Bcl-2, Bax, Bak, Bcl-x and Mcl-1 (21). Despite the similar expression of these proteins the L cells undergo apoptosis in response to staurosporine whereas the M cells are more resistant. This suggests that differences in other apoptotic regulatory pathways must exist between these two cell variants not accounted for by the expression of the Bcl-2 family of proteins. Recent studies by De Vente *et al* concluded that alterations in the PKC pathway could modulate the decision of a breast cancer cell to undergo death or differentiation (47). Specifically, the overexpression of PKC-α in MCF-7 cells resulted in the conversion from a modest cytostatic effect observed in phorbol ester-treated parental MCF-7 cells, to death of MCF-7 cells expressing higher levels of PKC-α. Similarly the L variant constitutively expresses higher levels of PKC-α than does the MCF-7 M stock which is not sensitive to cell death by the protein kinase inhibitor, staurosporine.

A number of reports have implicated PKC as a regulator of apoptotic responses in cells (66,67). Because of the number of PKC isoforms that exist it is possible that induction of apoptosis in response to PKC inhibition may be determined by differential expression of individual isoforms of PKC. We show here that the M and L variants express differing amounts of the PKC isoforms α and δ. A report by De Vente *et al* showed that in MCF-7 cells, overexpression of PKC-α sensitized those cells to cell death in response to PKC down-regulation whereas the normally low PKC-α expressing cells remain viable (47). These results suggest that one mechanism capable of enhancing sensitivity of the L stock to staurosporine-induced apoptosis may be through its increased expression of PKC-α. Manni *et al* demonstrated that overexpression of PKC-α was associated with decreased proliferation (46), which is consistent with the observed high PKC-α levels in MCF-7 L variant and the observed lower basal rate of proliferation in these cells (21). However Ways *et al* demonstrated an increased proliferative rate and tumorigenicity with PKC-α overexpression (45). An attractive explanation would be that inherent differences among the variants used in these two reports could lead to vastly different cellular responses with PKC-α overexpression.

Variations in estrogenic response and expression of estrogen-regulated genes have been previously reported in MCF-7 cell variants (3-5). Additionally, we along with other groups have shown that estrogen treatment of MCF-7 cells inhibits cell death induced by TNF, tamoxifen and doxorubicin (14,26,28,30). Although these reports also suggest that this effect is through increased expression of the Bcl-2 gene, estrogenic pathways may potentially regulate other anti-apoptotic genes. Thus, the lower maximal estrogenic response and basal estrogenic activity of the L variant as compared to the M variant may account for a greater sensitivity of the L variant to apoptosis induced by staurosporine under the culture conditions used. This reduced estrogenic activity of the L variant may be in part due to the lack of ERß expression. In general PKC overexpression in breast cancer cells coincides with enhanced oncogenicity as well as multi-drug resistance (44,45,68). Additionally, overexpression of the PKC-α isoform in MCF-7 cells is associated with a decreased estrogen response (69). This suggests that the decreased estrogen response of the MCF-7 L variant may also be influenced by the increased PKC-α expression. Recently PKC-δ expression in MCF-7 cells has been demonstrated to be regulated by estrogen treatment (70). The decreased estrogen response in the MCF-7 L variant may also lead to subsequent altered levels of PKC-δ in these cells. MCF-7ADR cells have been previously demonstrated to be resistant to TNF- and chemotherapeutic drug-induced apoptosis (71,72). Additionally, other studies have demonstrated a lack of estrogen response in MCF-7ADR cells suggesting that apoptotic resistance may be associated with progression to an ERα independent phenotype (54,55). It is interesting that despite the presence of ERß these cells have been previously demonstrated to be ERα negative and estrogen insensitive.

Our results in combination with these previously observed links between PKC and estrogen signaling suggest that these two pathways are intimately involved in regulation of each other's activity. Disturbing the balance between ER signaling and PKC expression or activation may lead to significant alteration in cellular responses such as proliferation, differentiation and apoptotic sensitivity. We describe unique differences in apoptotic sensitivity, estrogen response and PKC expression among MCF-7 cell variants, suggesting the importance of cellular context of the MCF-7 cell type studied. However, our observations may also offer insights regarding MCF-7 cell variants as a model of cancer cell progression to drug resistance and estrogen independence.

Our results suggest a potential molecular basis for differences in susceptibility to apoptosis among MCF-7 breast cancer cell variants. Previously we demonstrated

that differences in Bcl-2 and ceramide generation in these MCF-7 breast cancer cell variants could partially account for susceptibility to TNF-induced apoptosis. We also predict constitutive variations of estrogenicity and PKC isoform expression to be part of the cell survival/death signaling pathways that vary between cell phenotypes and determine cell fate in response to apoptotic stimuli. These differences may in part be due to constitutive variations in expression of members of the Bcl-2 family of proteins, as well as variations in estrogenicity and PKC isoform expression.

Acknowledgments

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Estrogenic and Antiestrogenic Activities of Flavonoid Phytochemicals Through Estrogen Receptor Binding-Dependent and -Independent Mechanisms

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Abstract: *Members of the flavonoid class of phytochemicals have previously been demonstrated to possess estrogenic activity in a number of hormonally responsive systems. We have performed the present study to characterize the estrogenic and antiestrogenic activity of flavonoids in the estrogen receptor (ER)-positive MCF-7 human breast cancer cell line. Using an ER-dependent reporter gene assay and an ER competition binding assay, we have identified phytochemicals possessing estrogenic and antiestrogenic activities, which appeared to correlate directly with their capacity to displace [^{3}H]estradiol from ER. Several flavonoids, including kaempferide, apigenin, and flavone, were distinct, in that their antiestrogenic activity did not appear to correlate with binding to ER, and therefore their suppression of estrogen-mediated gene transactivation and proliferation may occur independent of direct antagonism of the receptor. Further examination in HEK-293 cells transfected with ERα or ERβ demonstrated potent antagonism with kaempferide and apigenin, while flavone was weakly antagonistic only toward ERβ. These results suggest that the receptor binding-independent antiestrogenic chemicals may function through alternate signaling pathways as indirect ER modulators in a receptor- and cell type-specific manner. We conclude that antiestrogenic activities of flavonoid phytochemicals may occur through ER binding-dependent and -independent mechanisms and that the binding-independent antiestrogen activity of certain flavonoids is biologically significant in regulation of breast cancer cell proliferation.*

Introduction

Phytochemicals are produced by a wide variety of plants and function as insect deterrents and fungicides, with some responsible for initiating the symbiotic relationship between legumes and nitrogen-fixing bacteria (1–3). Numerous reports have implicated flavonoid phytochemicals as possessing hormone-disrupting activity, in particular acting as environmental estrogens (3–6). The endocrine-disrupting effects of flavonoids are seen in examples of sheep grazing on flavonoid-rich clover, and cheetahs fed soy-rich diets have presented with infertility, reproductive abnormalities, and tumors (3,7,8). In contrast to these detrimental effects of flavonoids, some epidemiological evidence has suggested that populations that consume soy-rich diets high in certain isoflavones display a significantly lower incidence of hormone-dependent, e.g., breast and prostate, cancer (9–12). The hormonal effects of the flavonoids and other phytochemicals such as stilbenes and lignans, as well as the fungal-derived zearalenones, may be mediated through regulation of endogenous hormone levels, alteration in steroid metabolism, or direct interaction with hormone receptors (5,6,13,14). Additionally, flavonoids have been shown to suppress carcinogenesis in numerous tissue types and systems, an effect that may depend on these chemicals' hormonal activity (1–6, 15–17).

Numerous phytochemicals have been shown to have estrogenic activity by *in vitro* and *in vivo* assays, and their estrogenic activity, particularly that of soy isoflavones, is consistent with the prevention of osteoporosis and cardiovascular disease in human populations (3–6,18,19). Coumestrol and genistein have been shown to increase uterine weight in mice (20), a classic measure of estrogenic activity *in vivo*. These chemicals have been shown to interact with and activate the estrogen receptor (ER) expressed in yeast and mammalian cells (21,22). Other phytochemicals have been shown to display estrogenic activity by *in vitro* assays. For example, narigenin, apigenin, phloretin, β-zearanol, zearalenone, and biochanin A increased estrogen-regulated gene transcription in HeLa cells transiently transfected with ERα (23). Additionally, several phytochemicals have been shown to be estrogenic as measured by their ability to stimulate the growth of MCF-7 cells (21,24). The recent identification of ERβ suggests another potential target for phytochemical action in estrogen signaling (25). In fact, certain phytochemicals possess differential affinity for and trans-

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activation of ERβ vs. ERα (26,27). The selective affinity and tissue-specific localization may further explain the ability of certain phytochemicals to affect tissues not previously demonstrated to contain ERα.

In contrast to their estrogenic activity, some phytochemicals have also been shown to possess antiestrogenic activity by suppressing estrogen-mediated transcription or proliferation. However, studies examining the antiestrogenic activity of phytochemicals have not been as extensive as those analyzing their estrogenic activity. Genistein, a known tyrosine kinase inhibitor, has been demonstrated to act in a concentration-dependent manner as an estrogen or an antiestrogen, as measured by reporter gene assay or proliferation of MCF-7 cells (28–30). However, the ability of genistein to inhibit proliferation of ER-negative breast cancer cells suggests that some effects may be mediated through ER-independent mechanisms (31–33). An early report demonstrated that coumestrol and genistein inhibited the uterovaginotrophic activity of estradiol, estrone, and diethylstilbestrol in mice (20). However, it was reported that coumestrol and genistein, along with biochanin A and zearalenone, did not affect estradiol-dependent proliferation of MCF-7 cells (24). Other studies have shown that enterolactone and narigenin inhibited estrogen-stimulated proliferation of MCF-7 cells (34), with narigenin also inhibiting the activity of 17β-estradiol in whole animal studies (35). More recent results have demonstrated a role for a number of other phytochemicals, including quercitin and luteolin, in inhibition of proliferation of MCF-7 cells (29,36,37). Given the potential role of phytochemicals in decreasing the risk of certain hormone-dependent cancers, the regulation of estrogen-mediated responses by phytochemicals (i.e., antiestrogenicity) may have important human health implications.

We previously used human ERα expressed in yeast to characterize the antiestrogenic and estrogenic activities of various phytochemicals (22). This study identified three classes of phytochemicals on the basis of their antiestrogenic and estrogenic activities. The first class, which included coumestrol and genistein, displayed estrogenic, but no antiestrogenic, activity. The second class exhibited minimal estrogenic activity and complex antiestrogenic activity that was dependent on the concentration of the phytochemical. This class was comprised of narigenin, apigenin, and kaempferide. The third class, which included biochanin A, flavone, and chrysin, had minimal estrogenic activity but functioned as dose-dependent antiestrogens.

Given the incomplete understanding of the estrogenic and antiestrogenic activity of flavonoids and other phytochemicals, we examined the ability of a number of flavonoid phytochemicals to alter 17β-estradiol-mediated transcriptional activity in human cells. This systematic characterization of the antiestrogenic and estrogenic, receptor binding, and cell proliferative activities of flavonoids in a relevant cancer cell line will allow us to further understand the role of these compounds in human health. Here, we demonstrate that phytochemicals can be grouped into three classes on the basis of their antiestrogenic activity in a reporter gene assay and by their binding to ERs in MCF-7 cells. Some phytochemicals function as estrogens without antiestrogenic activity. Those phytochemicals with antiestrogenic activity appeared to exert their effects through ER binding-dependent and -independent pathways. The ability of certain phytochemicals to act as antiestrogens independent of ER binding suggests that alternate signaling pathways exist by which flavonoids function to suppress steroid hormone/receptor action.

Materials and Methods

Materials

17β-Estradiol, phloretin (2′,4,4′,6′-tetrahydroxychalcone), genistein (5,7,4′-trihydroxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), α-zearalenol, β-zearalenol (2,4-dihydroxy-6[6*B*,10-dihydroxy-*trans*-1-undecenyl]benzoic acid *m*-lactone), chrysin (5,7-dihydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), narigenin (4′,5,7-trihydroxyflavanone), zearalenone, and flavone (2-phenyl-4*H*-1-benzopyran-4-one) were purchased from Sigma Chemical (St. Louis, MO). Coumestrol (3,9-dihydro-6*H*-benzofuro[3,2-*c*]-[1]benzopyran-6-one) was purchased from Acros Organics (Geel, Belgium). Kaempferol (3,4′,5,7-tetrahydroxyflavone) and kaempferide (4-methoxy-3,5,7-trihydroxyflavone) were purchased from Fluka Biochemika (Buchs, Switzerland). Luteolin (3′,4′,5,7-tetrahydroxyflavone) and 7,4′-dihydroxyflavone were purchased from INDOFINE Chemical (Somerville, NJ). All chemicals were prepared in dimethylsulfoxide and added to the media so the final concentration of solvent did not exceed 1%. 17β-3,4,6,7[^{3}H](N) estradiol (99 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE).

Cell Culture

MCF-7 cells (M variant p250) were cultured in 150-cm^2 culture flasks in Iscove's modified Dulbecco's medium, and human embryonic kidney (HEK)-293 cells were cultured in 150-cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM). Both types of media were supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL, Gaithersburg, MD), as well as basic minimal essential and minimum essential medium amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (diluted in the medium to a 1× concentration from 100× or 50× stocks), as well as 10^{-8} M porcine insulin (Sigma Chemical). The culture flasks were maintained in a cell incubator at a humidified atmosphere of 5% CO_2-95% air at 37°C.

Luciferase Assays

As previously described (38), MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% DCC-FBS) for 48 hours before they were plated. The cells were plated in six-well plates at 1×10^6 cells/well in the same media and allowed to

attach overnight. On the next day, the cells were transfected for five hours in serum/supplement-free DMEM with 2 μg of pERE2-luciferase plasmid, which contains two copies of the vitellogenin ERE linked to the luciferase gene and 1 μg of the pCMVβ-galactosidase plasmid using 9 μl of Lipofectamine (GIBCO-BRL). HEK-293 cells were plated in 12-well plates at 5×10^5 cells/well in 5% DCC-FBS and allowed to attach overnight. On the next day, the cells were transfected for five hours in serum/supplement-free DMEM with 1 μg of pERE2-luciferase plasmid and 500 ng of pCDNA3.1-ERα or 10 ng of pCDNA3.1-ERβ. After five hours, the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle, 17β-estradiol, phytocompound, or 17β-estradiol + phytocompound and incubated for 18–24 hours at 37°C.

The treatment-containing medium was removed, and 250 μl of 1× lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) were added per well and incubated for 15 minutes at room temperature. The cell debris was then pelleted by centrifugation at 15,000 *g* for five minutes. The cell extracts were normalized for protein concentration using the Bio-Rad reagent following the supplied protocol (Bio-Rad Laboratories, Hercules, CA). For β-galactosidase assays, the cell extract was placed in 500 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 35 mM β-mercaptoethanol), 100 μl of *o*-nitrophenyl-β-D-galactopyranoside at 4 mg/ml in Z buffer were added to each reaction, and the tubes were maintained at 37°C. The addition of 400 μl of 1 M Na_2CO_3 terminated the reactions. The β-galactosidase activity of each reaction was measured at an absorbance of 420 nm. Luciferase activity for the cell extracts was determined using luciferase substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). The data are the result of at least three independent experiments with three replicates each. Statistically significant ($p < 0.05$) increases or decreases in percent 17β-estradiol activity were determined using single-factor analysis of variance (Microsoft Excel).

[^{3}H]17β-Estradiol Competition Binding Assays

MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% DCC-FBS for 72 hours before the binding assay. Cells were washed with phenol red-free DMEM and resuspended in the binding buffer (phenol red-free DMEM + 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4) at 1×10^6 cells/ml. Each reaction received 1 ml of cell resuspension and 1 nM [^{3}H]17β-estradiol in the presence or absence of 150-fold molar excess of radioinert 17β-estradiol or 1, 10, or 25 μM radioinert phytocompounds for two hours at 37°C. After incubation, the reactions were centrifuged at 5,000 *g* for four minutes, and the cells were washed as follows: one cold phosphate-buffered saline (PBS) wash, followed by one 5% charcoal-stripped serum wash, and then two additional cold PBS washes. Cell pellets were then resuspended in 100 μl of cold PBS, and bound [^{3}H]17β-estradiol was measured in a scintillation counter. The data are the results of at least two independent experiments each containing three replicates. Statistically significant increases or decreases in percent [^{3}H]17β-estradiol bound were determined using single-factor analysis of variance (Microsoft Excel).

Proliferation Assays

MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% DCC-FBS for 48 hours before they were plated. Cells were plated at a density of 5×10^4 cells per 35-mm well and maintained at 37°C for an additional 24 hours in the phenol red-free DMEM supplemented with 5% DCC-FBS. The medium was replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle, 17β-estradiol, or 17β-estradiol + 100 nM 4-hydroxytamoxifen, 25 μM apigenin, 25 μM flavone, or 25 μM kaempferide. On the 1st, 3rd, and 5th days of treatment, the cells were removed from the plate with PBS-EDTA and counted using a hemocytometer. The data are the results of at least two independent experiments each containing three replicates.

Results

Estrogenic Activity of Phytochemicals in MCF-7 Cells

MCF-7 human breast cancer cells, which express predominantly ERα and weakly express ERβ (39–44), were used to examine the estrogenic activity of a number of phytochemicals (Figure 1). The estrogenic activities were measured by transfection with an estrogen-sensitive luciferase plasmid and treatment with vehicle, estradiol, or the phytochemicals. Luciferase activity was maximal with 1 nM estradiol in MCF-7 cells (Figure 2, top). Because the binding affinity of most phytochemicals is ≥1,000-fold lower than that of estradiol (21–23,27,45), we chose to test concentrations that were 1,000-, 10,000-, and 25,000-fold greater than estradiol. The most effective chemical tested was chrysin, which had ~125% of the activity of 1 nM estradiol at 10 and 25 μM (Figure 2). Biochanin A at 10 μM had activity equivalent to 1 nM estradiol, but at 1 and 25 μM the activity was lower (Figure 2). The only phytochemicals that did not display significant estrogenic activity were flavone (Figure 2) and kaempferol (data not shown).

Two types of dose-response relationships were observed for the estrogenic activity of phytochemicals in MCF-7 cells: 1) a sigmodial dose-response curve was observed for the estrogenic activity of chrysin, phloretin, and narigenin (Figure 2), and 2) a convex dose-response curve was observed for other phytochemicals and estradiol. This curve did not display a linear relationship between luciferase activity and chemical concentration. For example, genistein had ~85% of the activity of 1 nM estradiol at 1 and 10 μM and only 30% of the activity of 1 nM estradiol at 25 μM (Figure

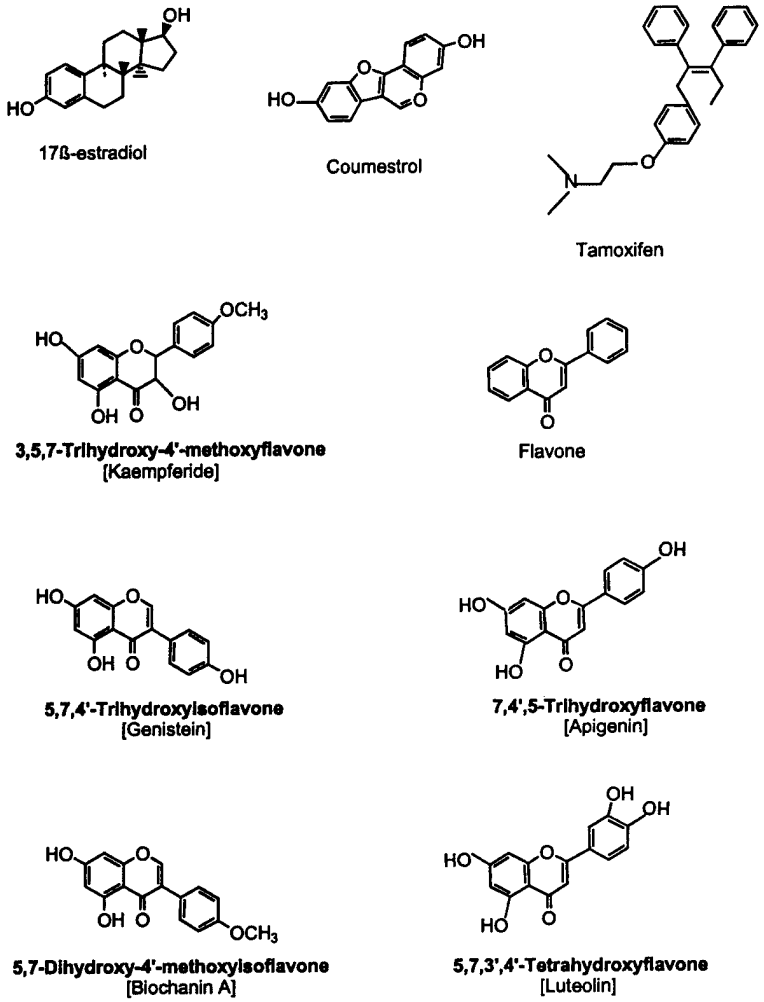


Figure 1. Structure of 17β-estradiol, 4-hydroxytamoxifen, and selected flavonoids.

2). In addition, apigenin, kaempferide (Figure 2), and biochanin A (Figure 2) had maximal luciferase activity at 10 μM and lower activity at 1 and 25 μM. This type of activity was also observed for α-zearanol, zearalenone, luteolin, β-zearanol, and 7,4′-dihyroxyflavone (Figure 2).

Antiestrogenic Activity of Phytochemicals in MCF-7 Cells

The antiestrogenic activities of the various phytochemicals were measured using MCF-7 cells transfected with the estrogen-sensitive luciferase plasmid and incubated with 1 nM estradiol in the presence or absence of the phytochemicals at 1, 10, or 25 μM (Figure 3). The ability of this system to examine the antiestrogenic activity of chemicals was validated with the antiestrogen 4-hydroxytamoxifen (Figure 3). As expected, 100 nM 4-hydroxytamoxifen reduced estradiol-induced luciferase activity by 93%. Additionally, the ability of 4-hydroxytamoxifen to reduce the estrogenic activity of a phytochemical from each group was assessed. The estrogenic activity of coumestrol and biochanin A at 1 μM was inhibited by ~93% as well (Figure 3). Kaempferide (Figure 3) did not display significant estrogenic activity, and therefore the ability of 4-hydroxytamoxifen to inhibit its estrogenic activity was insignificant. The ability of 4-hydroxytamoxifen to inhibit the estrogenic activity of these phytochemicals suggests that the phytochemicals elicit their estrogenic activity through the ER.

One of the most effective chemicals in reducing estradiol-dependent luciferase activity was luteolin (Figure 3). Luteolin decreased estradiol activity by 80% at 10 μM and by 90% at 25 μM. Kaempferide, apigenin, and flavone at 25 μM reduced estradiol activity by 85%, 88%, and 70%, respectively (Figure 3). All the phytochemicals decreased estradiol-induced luciferase activity to some extent, except for kaempferol, which had no inhibitory activity (data not shown). The weakest antiestrogens were narigenin and biochanin A, which had significant inhibitory activity only at 25 μM (Figure 3).

Whole Cell ER Binding Assays

To determine the ability of the phytochemicals to bind to ER, we performed a whole cell binding assay using [^{3}H]estradiol. A 150-fold molar excess of radioinert estradiol de-

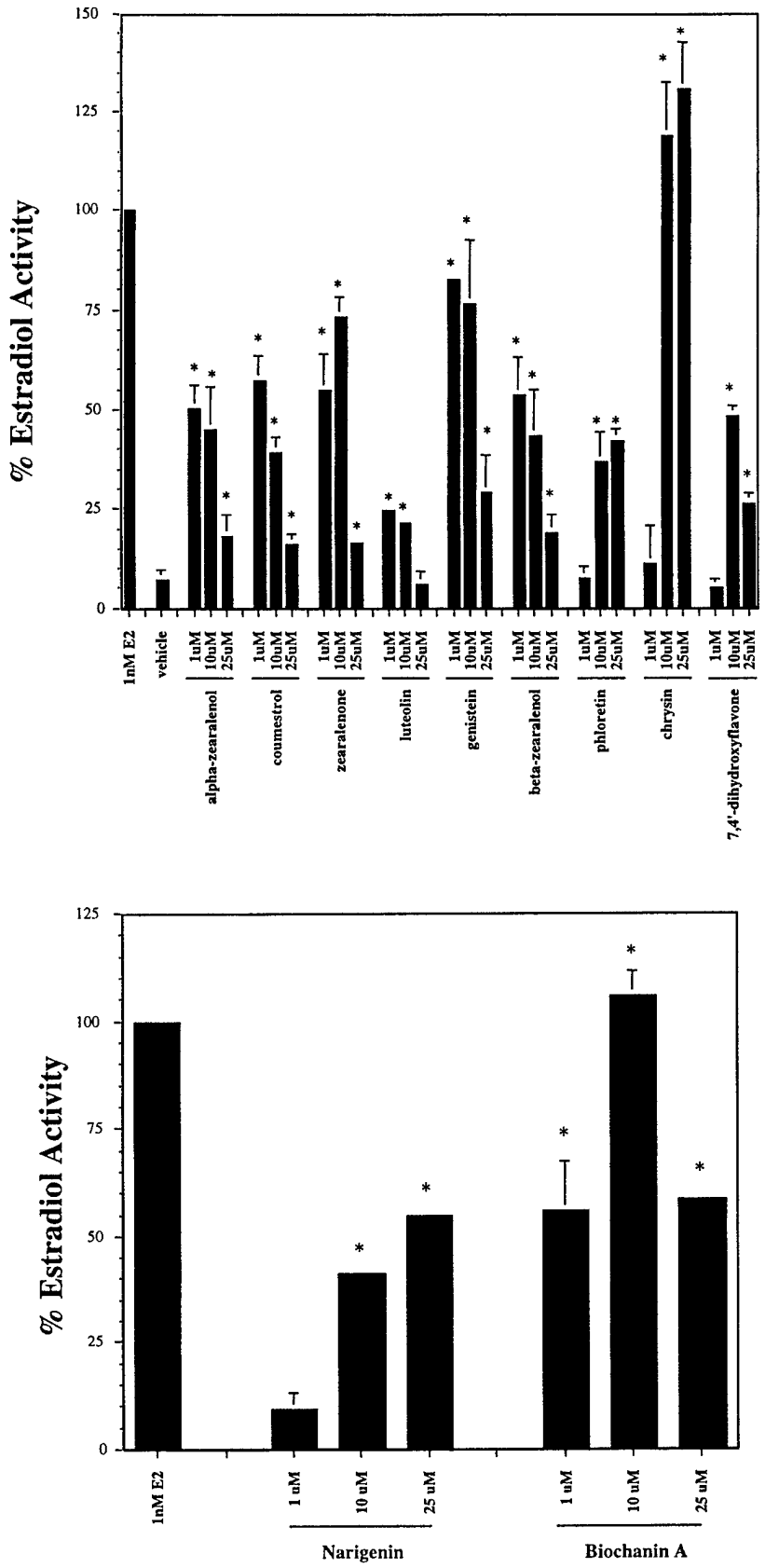


Figure 2. Estrogenic activity of phytocompounds in MCF-7 cells. MCF-7 cells were transfected with pERE2-luciferase and treated for 18 h with vehicle, 17β-estradiol, or indicated compounds at 1, 10, and 25 μM. Luciferase activity was measured as described in **Materials and Methods**. Relative light units (RLUs) obtained for 17β-estradiol were set as 100%, and RLUs of each phytocompound were calculated as a percentage of RLUs obtained for 1 nM 17β-estradiol. Values are results of ≥3 independent experiments with 3 replicates each. *, Significantly greater than vehicle. E2, 17β-estradiol.

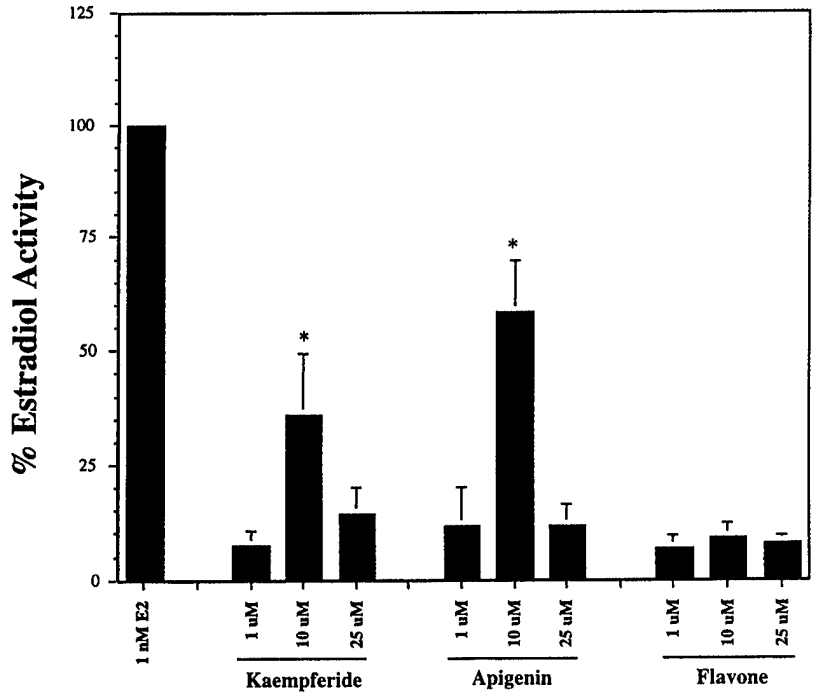


Figure 2. *(Continued)*

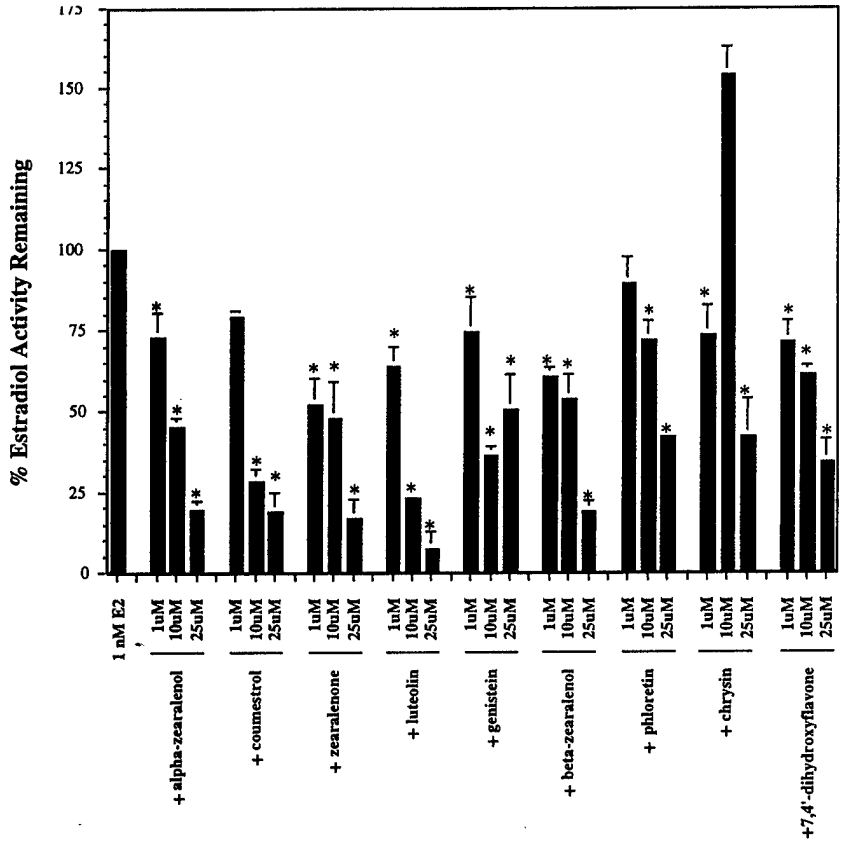
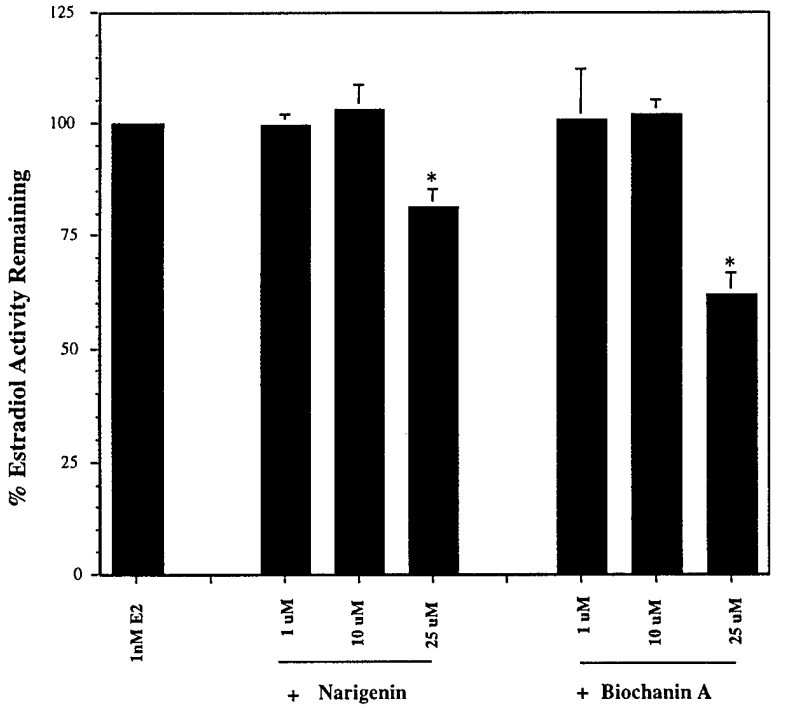


Figure 3. Antiestrogenic activity of phytocompounds in MCF-7 cells. First 3 panels: MCF-7 cells were transfected with pERE2-luciferase and treated for 18 h with vehicle, 17β-estradiol, or 17β-estradiol + indicated compounds at 1, 10, and 25 μM. Last panel: ability of this system to examine antiestrogenic activity of chemicals as validated with the antiestrogen 4-hydroxytamoxifen. Luciferase activity was measured as described in **Materials and Methods**. RLUs obtained for 17β-estradiol were set as 100%, and RLUs for each phytocompound or 4-hydroxytamoxifen + 17β-estradiol were calculated as a percentage of RLUs obtained for 17β-estradiol. Values are results of ≥3 independent experiments with 3 replicates each. *, Significant decrease in percent estradiol activity. Increase in percent estradiol activity for 10 μM chrysin is statistically significant ($p < 0.05$).



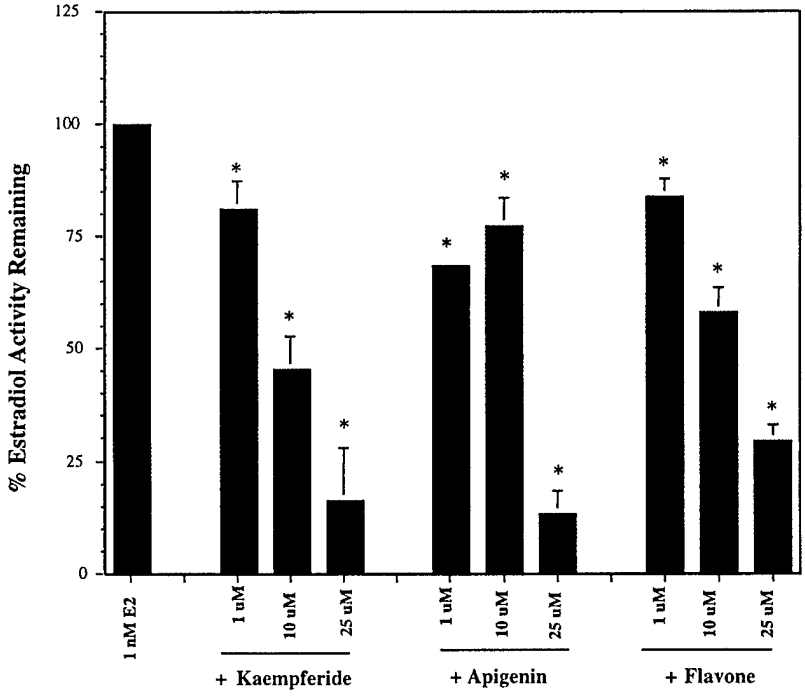


Figure 3. *(Continued)*

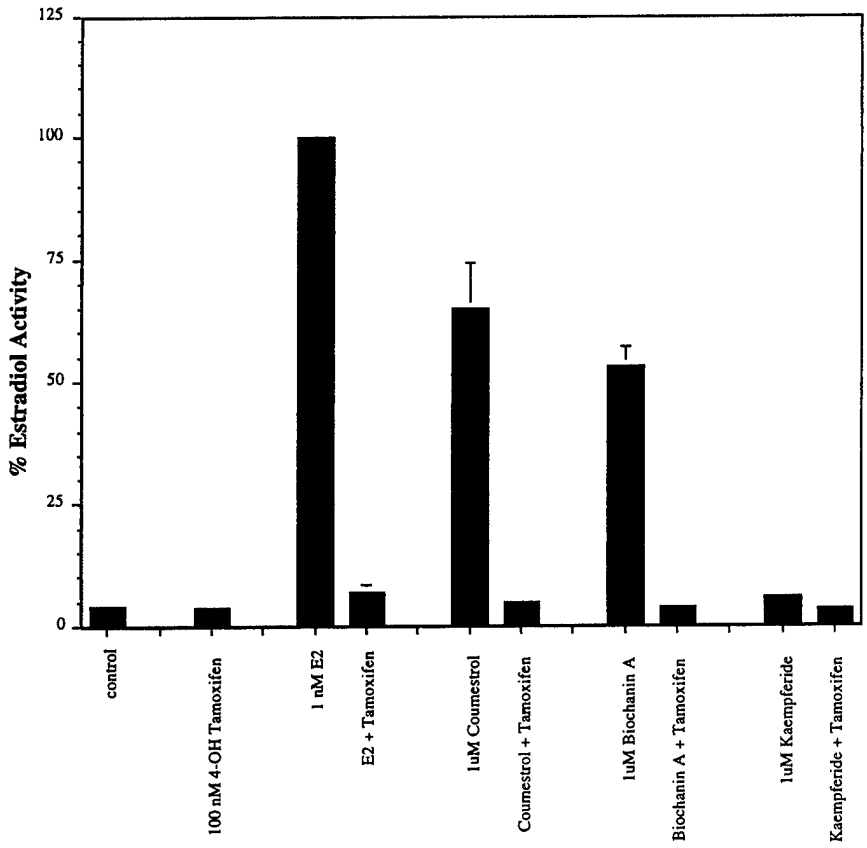


Figure 3. *(Continued)*

creased [^{3}H]estradiol binding by 80%, indicating specific binding of [^{3}H]estradiol binding to ER in MCF-7 cells (Figure 4). Several phytochemicals, such as coumestrol, α-zearalenol, genistein, and luteolin, effectively displaced [^{3}H]estradiol from the human ER (Figure 4). These results are consistent with studies measuring the binding of the same phytochemicals using *in vitro* assays (21–23,27,45). Other chemicals weakly reduced [^{3}H]estradiol binding to ER (Figure 4). Among these chemicals, kaempferide and apigenin had weak ER binding abilities (Figure 4). Flavone did not reduce [^{3}H]estradiol binding (Figure 4).

Classification of Phytochemicals in MCF-7 Cells

We grouped the phytochemicals into three classes on the basis of their antiestrogenic activities in the luciferase assays and their capacity to displace [^{3}H]estradiol from ER in whole cell binding assays (Table 1). The first class included the following phytochemicals: α-zearalenol, β-zearalenol, coumestrol, zearalenone, luteolin, genistein, phloretin, chrysin, and 7,4′-dihydroxyflavone. The strong antiestrogenic activity of chemicals in the first class appeared to correlate directly with their ability to inhibit [^{3}H]estradiol from binding to ER. The second class contained the phytochemicals narigenin and biochanin A. This class displayed minimal antiestrogenic activity and weakly interacted with ERs. The third class consisted of flavone, kaempferide, and apigenin. These phytochemicals displayed strong antiestrogenic activity; however, they were distinct from chemicals in the first class, since their antiestrogenic activity did not appear to correlate with binding to ERs.

Inhibition of Estrogen-Dependent Cell Growth

To determine the biological significance of the antiestrogenic activity displayed by the third class of phytochemicals in the luciferase assay, the capacity of these chemicals to inhibit the estradiol-induced proliferation of MCF-7 cells was measured. MCF-7 cells were treated with estradiol in the presence or absence of the antiestrogen 4-hydroxytamoxifen, kaempferide, flavone, or apigenin. Estradiol-dependent cell growth was completely inhibited by 0.1 μM 4-hydroxytamoxifen (Figure 5). Similar to tamoxifen, the phytochemicals completely blocked estradiol-induced cell growth. Chrysin and luteolin also had growth-inhibitory activity but were less effective than kaempferide, flavone, or apigenin at inhibiting estradiol-induced cell proliferation (Table 1).

Antiestrogenic Activity of Phytochemicals in HEK-293 Cells

The antiestrogenic activities of the various phytochemicals were measured using HEK-293 cells cotransfected with the estrogen-sensitive luciferase plasmid along with the hu-

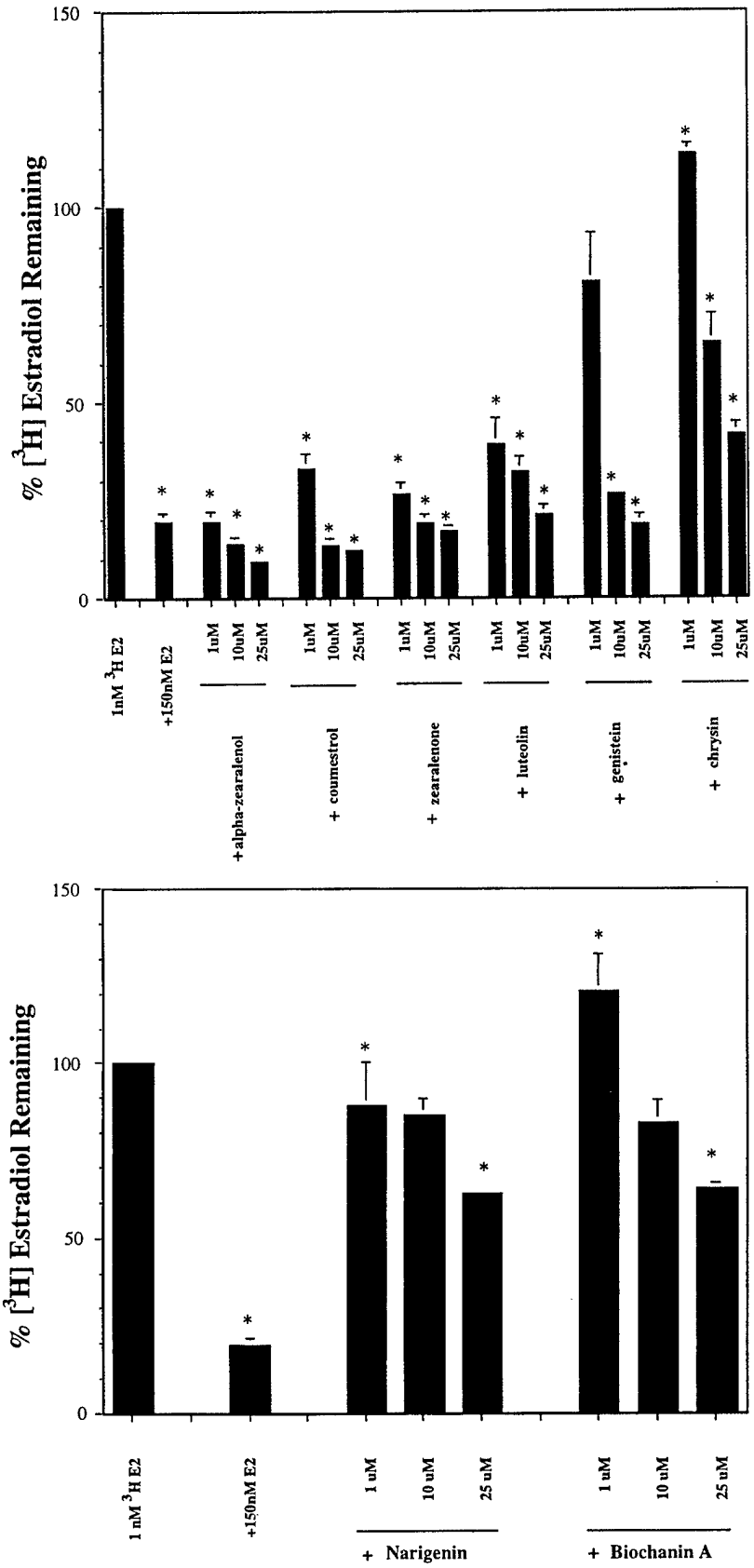


Figure 4. Competition binding assays in MCF-7 cells. Competition binding reactions were performed using 1×10^6 cells per reaction with 1 nM [^{3}H]17β-estradiol in presence or absence of 150-fold molar excess radioinert 17β-estradiol or 1, 10, and 25 μM radioinert phytocompounds for 2 h at 37°C. Bound [^{3}H]17β-estradiol was measured in a scintillation counter. Values are results of ≥2 independent experiments each containing 3 replicates. *, Significantly different from 17β-estradiol activity ($p < 0.05$).

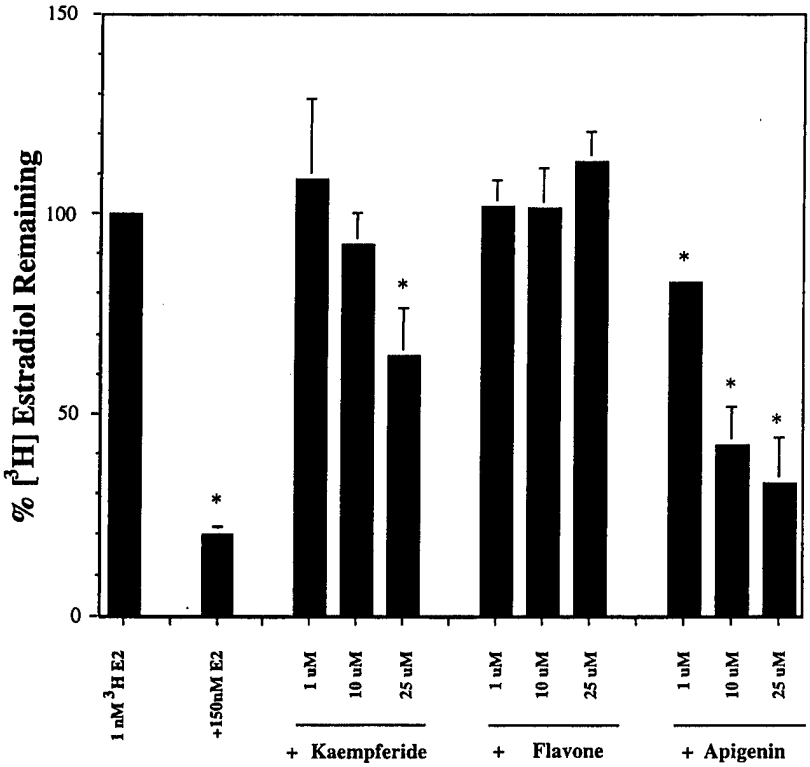


Figure 4. *(Continued)*

Table 1. Classification of Phytochemicals in MCF-7 Cells[a]

Compound	Estrogenic Activity[b]	Antiestrogenic Activity[c]	ER Binding[d]	Growth Inhibition[e]
17β-Estradiol	++++			
4-Hydroxytamoxifen		++++	++++	++++
α-Zearalenol	++	+++	++++	ND
β-Zearalenol	++	+++	ND	ND
Coumestrol	++	+++	++++	ND
Zearalenone	+++	+++	++++	ND
Luteolin	+	++++	++++	+++
Genistein	+++	++	++++	ND
Phloretin	++	++	ND	ND
Chrysin	++++	++	+++	+++
7,4′-Dihydroxyflavone	++	++	ND	ND
Narigenin	++	+	++	ND
Biochanin A	++++	+	++	ND
Kaempferide	+	+++	++	++++
Flavone	+	+++	+	++++
Apigenin	++	+++	+++	++++

a: Activities for each chemical were compared for their estrogenic activity (to 1 nM estradiol), antiestrogenic activity (to 100 nM tamoxifen), estrogen receptor (ER) competition binding (to cold estradiol), or inhibition of proliferation (100 nM tamoxifen). ND indicates compounds not tested.

b: ++++ is represented as equal to or greater than estradiol (1 nM) activity alone (100%). Compounds with weaker activities are represented as +++ (66–99%), ++ (33–66%), and + (0–33%) compared with estradiol alone (1 nm).

c: ++++ is representative of 90–100% suppression of estradiol's (1 nM) activity as seen with tamoxifen (100 nM). Compounds with weaker antiestrogenic activities are represented as +++ (60–90%), ++ (30–59%), and + (0–29%).

d: ++++ is representative of 75% reduction in [^{3}H]estradiol (1 nM) binding as seen with tamoxifen (100 nM) and cold estradiol (150 nM). Compounds with weaker ER competition binding are represented as +++ (50–74%), ++ (25–49%), and + (0–25%).

e: ++++ is representative of 100% suppression of estradiol (1 nM)-induced proliferation as seen with tamoxifen (100 nM). Compounds with weaker activity are represented as +++.

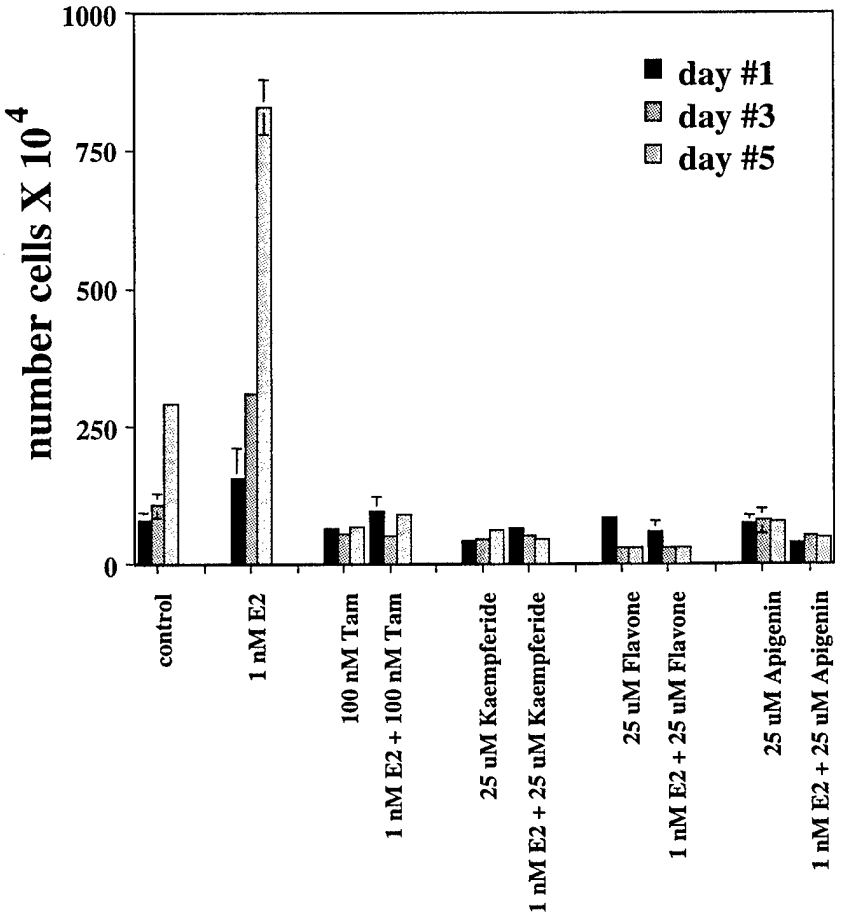


Figure 5. MCF-7 cell proliferation experiment. Cells were plated at a density of 5×10^4 cells per 35-mm well and treated with vehicle, 17β-estradiol, or 17β-estradiol + 100 nM 4-hydroxytamoxifen (Tam), 25 μM apigenin, 25 μM flavone, or 25 μM kaempferide. On 1st, 3rd, and 5th days of treatment, cells were removed from plate and counted using a hemocytometer. Values are results of ≥2 independent experiments each containing 3 replicates.

man ERα or ERβ and incubated with 1 nM 17β-estradiol in the presence or absence of the phytochemicals at 1, 10, or 25 μM (Figure 6). Treatment with 4-hydroxytamoxifen exhibited 8.7 ± 0.57% and 8.9 ± 1.0% of 17β-estradiol activity alone and 8.1 ± 0.93% and 15.6 ± 3.2% of activity in combination with 1 nM estradiol with ERα and ERβ, respectively. The ability of the binding-independent antiestrogen (BIA) phytochemicals apigenin, flavone, and kaempferide was examined using this system to further evaluate their antiestrogenic effects. In this system, with ERα or ERβ, kaempferide possessed no estrogenic activity at 25 μM and was capable of suppressing 17β-estradiol-mediated luciferase activity to 8.9 ± 3.4% and 8.1 ± 2.4% of 17β-estradiol alone. Similar to the results obtained with MCF-7 cells, apigenin was weakly estrogenic and was capable of suppressing 17β-estradiol-stimulated activity by 31.9 ± 10% and 21.7 ± 7.8% with ERα and ERβ, respectively. Consistent with previous findings of Kuiper and co-workers (27) in HEK-293 cells and our results with MCF-7 cells, flavone did not possess any ERα or ERβ agonistic activity. In contrast to the results obtained in MCF-7 cells, flavone was unable to inhibit 17β-estradiol activity with ERα (132.3 ± 26.3%) and only marginally (64.9 ± 6.9%) suppressed 17β-estradiol activity with ERβ in HEK-293 cells. These contrasting results suggest that certain BIAs function in a cell type-specific manner and may function to selectively antagonize specific ERs.

Discussion

The term phytoestrogen has often been used to refer to those plant compounds that possess agonistic activity on the ER. These effects of phytoestrogens may be mediated through direct interaction with the ER or potentially through binding-independent activation of other signaling pathways that converge on the ER to enhance or stimulate activity (46). In particular, the phytoestrogens most often examined are those isoflavones found in soybeans and soy foods. However, many other phytochemicals, including flavonoids, saponins, phytostilbenes, and lignans, as well as fungal compounds, have also been shown to possess estrogenic activity in a number of systems. The agonistic activities of phytochemicals can partially explain some of the observed beneficial effects of soy on osteoporosis and cardiovascular disease. However, the epidemiological link between high soy/flavonoid consumption and a decreased risk of breast

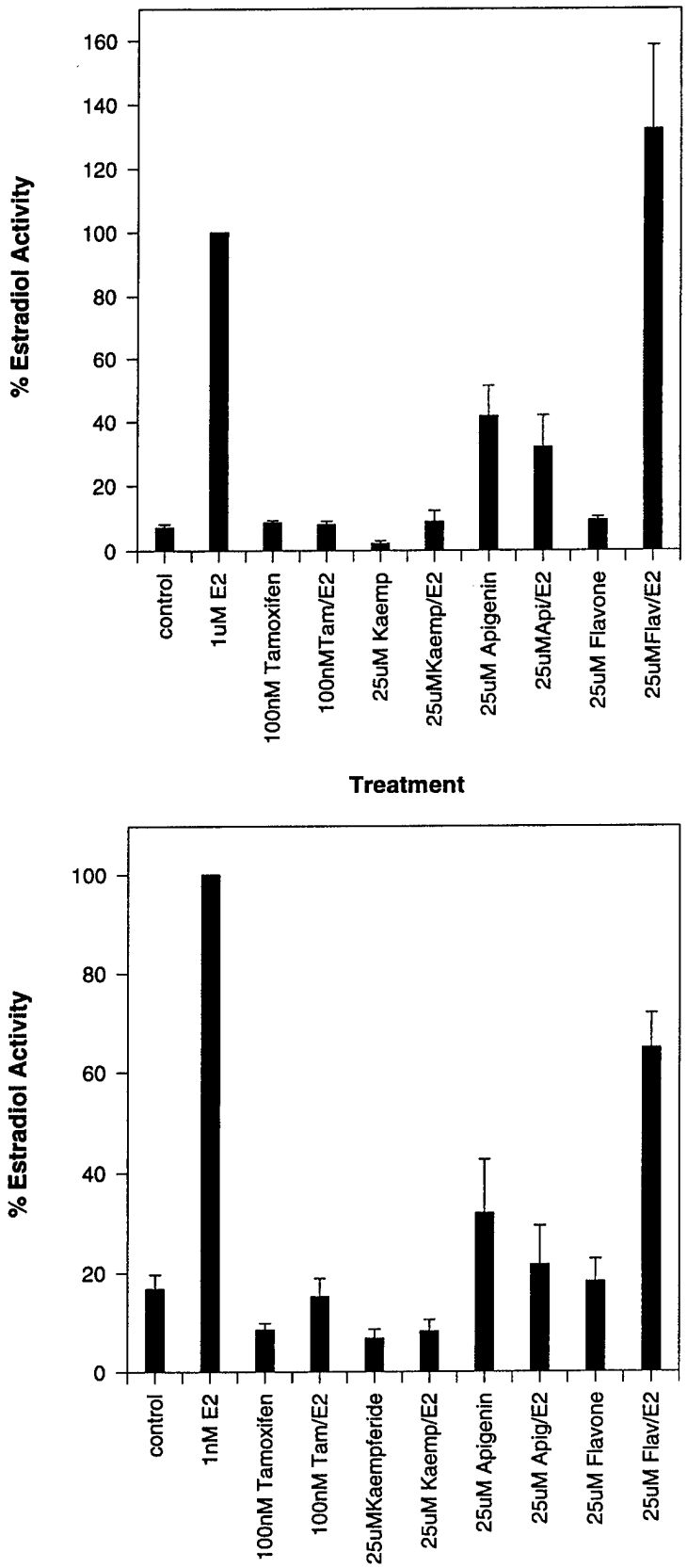


Figure 6. Estrogenic activity of phytocompounds in HEK-293 cells. Cells were cotransfected with either pCDNA3-ERα (top) or pCDNA3-ERβ (bottom) and pERE2-luciferase, then treated for 18 h with vehicle, 1 nM 17β-estradiol, or indicated compounds at 25 μM in presence or absence of 1 nM 17β-estradiol. Luciferase activity was measured as described in **Materials and Methods**. RLUs obtained for 17β-estradiol were set as 100%, and RLUs of each phytocompound were calculated as a percentage of RLUs obtained for 1 nM 17β-estradiol. Values are results of ≥4 independent experiments performed in duplicate. Kaemp, kaempferide; Api, apigenin; Flav, flavone.

cancers, as well as suppression of mammary carcinogenesis and diethylstilbestrol-induced developmental defects in animal models, suggests a potential antiestrogenic role for some of these chemicals. This study was performed to examine agonistic and antagonistic effects of a variety of phytochemicals with the human ERα.

We have grouped phytochemicals into three classes on the basis of their ability to function as antiestrogens and estrogens through ERα in MCF-7 human breast carcinoma cells. The first and second classes of phytochemicals displayed estrogenic activity, but only the first class exhibited strong antiestrogenic activity compared with the antiestrogen 4-hydroxytamoxifen (Table 1). For the most part, the antiestrogenic activity of these chemicals appeared to correlate with their binding to the ER and are referred to as binding-dependent antiestrogens (BDA). The phytochemicals in the third class had minimal estrogenic activity but substantial antiestrogenic activity. Whole cell binding studies suggested that the antiestrogenic activity of this class was not primarily mediated in an ERα binding-dependent manner; therefore, these compounds are referred to as BIAs. Additionally, the third class inhibited estradiol-induced growth to the same extent as the antiestrogen 4-hydroxytamoxifen.

The compounds in the first class (coumestrol, luteolin, genistein, phloretin, chrysin, and 7,4′-dihydroxyflavone) exhibited dose-dependent estrogenic and antiestrogenic effects. The ability of genistein and other phytochemicals to exert antiestrogenic effects at higher concentrations may be partially explained by their weak agonistic activity and ability to compete with estradiol for receptor binding. However, despite the correlation of ERα binding to antiestrogenicity, these chemicals may also function through alterations within alternate signaling pathways to affect ER activity. For example, genistein, a known tyrosine kinase inhibitor, at higher concentrations may function to suppress 17β-estradiol-dependent activation of tyrosine kinase receptors such as epidermal growth factor (EGF) receptor (32). A decrease in EGF receptor activity in the presence of genistein could reduce the activity of mitogen-activated protein kinase (MAPK) and other EGF-stimulated kinases or proteins. EGF and subsequent MAPK activation has been shown to phosphorylate ERs and, in some instances, is required for maximal ERα-mediated transactivation (47–49). The similar observation of dose-dependent antiestrogenicity by other phytochemicals may also be partially explained through additional regulation of cellular targets distinct from the ER. The estrogenic activity of other phytochemicals from the first group are consistent with previous reports demonstrating that these compounds function as ER agonists in HeLa cells transfected with ERs (21,23). Of interest is the observation that some phytochemicals function as estrogens in the absence of apparent ER binding. The observed ER binding-independent estrogenicity of biochanin A is consistent with observations by Kuiper and co-workers (27) in HEK-293 cells. This may be a function of the ability of biochanin A to activate cellular pathways that lead to enhanced ER function. However, the ability of biochanin A to be demethylated to genistein suggests that metabolism of phytochemicals to more potent estrogens or antiestrogens may represent an important function in their activity (50). In studies by another laboratory, narigenin was shown to function as an antiestrogen in MCF-7 cells and whole animal studies. The antiestrogenic activity of narigenin in the MCF-7 cells used in our study was much weaker than the activity reported by Ruh and colleagues (35).

From the beginning of the study, our aim was to identify phytochemicals with minimal estrogenic but potent antiestrogenic activity. The third class, consisting of apigenin, kaempferide, and flavone, represented phytochemicals with this type of activity. In yeast, flavone also had no estrogenic activity (22). Apigenin and kaempferide also had weak estrogenic activity in yeast and MCF-7 cells. Kaempferide, apigenin, and flavone functioned as antiestrogens in the luciferase and growth inhibition assays. Competition binding studies, however, demonstrated that kaempferide and apigenin weakly interacted with ERα and that flavone did not bind ERα. These results indicate that the antiestrogenic activity of this class of chemicals does not correlate with their interaction with ERα. An explanation for the BIA activity is that these chemicals might regulate proteins in cell signaling pathways. The capacity of flavone to inhibit the proliferation of cells lacking ER suggests that flavone exerts its activity through proteins other than ERs (51). Flavone and apigenin have been shown to inhibit the tyrosine kinase activity of EGF receptor but at a concentration ≥30- to 50-fold higher than genistein (32). Additionally, apigenin functions to inhibit the MAPK cascade in *ras*-transformed NIH 3T3 cells (52). The previously described role of apigenin and flavone in regulation of the MAPK cascade pathway offers an attractive potential target of BIA action. Apigenin, along with the synthetic 2′-amino-3′-methoxyflavone (PD-98059), has been shown to block growth factor- and phorbol ester-stimulated mitogen-activated extracellular signal-regulated kinase activation and subsequent extracellular signal-regulated kinase activity (53), and previous reports have described a cross talk between steroid hormones and activator protein-1 transcription factors (54,55). Alternatively, the flavone derivative flavopiridol inhibits the kinase activity of cyclin-dependent kinases-2 and -4, suggesting additional targets for phytochemicals (56).

Further evaluation of the BIA of flavones, apigenin, and kaempferide was performed in HEK-293 cells transfected with ERα. Consistent with the observations in MCF-7 cells, kaempferide and flavone possessed minimal agonistic activity, while apigenin was weakly agonistic. Recently, Kuiper and co-workers (27) used transiently transfected HEK-293 cells to examine the estrogenic activity of flavonoids on ERα and ERβ. Our results using these flavonoids are consistent with the minimal activity of apigenin and lack of estrogenicity of flavone in this system. Use of HEK-293 cells to examine antiestrogenic effects of flavonoids revealed that, consistent with the results in MCF-7 cells, apigenin and

kaempferide possessed strong antagonistic effects. In contrast to the observations in MCF-7 cells, flavone was not capable of suppressing 17β-estradiol activity in HEK-293 cells with ERα and only slightly suppressed ERβ activity. The differences between these two systems are suggestive of cellular differences that may account for the presence or absence of other signaling pathways that function to prevent or override those BIAs, such as flavone. These results may also be representative of the artificial system used to examine the antiestrogenic effect. Previous studies with yeast and HeLa cells demonstrated hyperbolic dose-response curves with a lack of biphasic effect at higher concentrations of phytochemicals. This is in contrast to our results with the endogenously hormone-responsive MCF-7 cells that displayed estrogenicity at lower concentrations and potent antiestrogenic effects at higher concentrations. The inability of certain phytochemicals to exert antiestrogenic effects at higher concentrations may represent a limitation of these systems or may function as an example of tissue-specific effects of estrogen signaling. The inability of artificial transfectants to recapitulate normal ER physiological effects, such as 17β-estradiol-induced proliferation observed in MCF-7 cells, may also account for the observed differences in estrogenic signaling between these cells, HEK-293 cells, and HeLa cells.

Our findings also allow us to speculate on structural features of flavonoid chemicals as they relate to estrogenic and antiestrogenic activity. In regard to estrogenic activity, increasing hydroxylation of the flavonoids at the 3′ and 4′ position is associated with decreased estrogenic activity: chrysin (5,7-OH-flavone) > apigenin (5,7,4′-OH-flavone) = narigenin (5,7,4′-OH-flavanone) = 7,4′-hydroxyflavone > kaempferide (5,7-OH-4′-methoxyflavone) = luteolin (5,7,3′4′-OH-flavone). In contrast, the ability of the flavonoids to suppress estrogen activity was directly correlated with increased hydroxylation or methoxylation of the 3′ and 4′ positions: luteolin (5,7,3′4′-OH-flavone) > kaempferide (5,7-OH-4′-methoxyflavone) = apigenin (5,7,4′-OH-flavone) > 7,4′-hydroxyflavone = chrysin (5,7-OH-flavone) > narigenin (5,7,4′-OH-flavanone). The weak estrogenic and antiestrogenic activity of the flavanone narigenin, compared with the correspondingly hydroxylated flavone apigenin, may be due to the lack of a 2-3 double bond, suggesting an important role for unsaturated carbons at these positions.

The relation between hydroxylation and estrogenic or antiestrogenic activity is not seen with the unhydroxylated compound flavone. However, flavone, a potent antiestrogenic chemical, was not observed to compete with estradiol for binding, suggesting ER binding-independent mechanisms. Regardless of their ability to directly compete with estradiol for ER binding or their putative BIA or BDA activity, the hydroxylation status of the flavonoids described closely correlates to antiestrogenic activity and inversely correlates with estrogenic activity.

In conclusion, we have grouped phytochemicals into three classes on the basis of their antiestrogenic or estrogenic and human ER binding activity. This study highlights the broad range of antiestrogenic or estrogenic activities exhibited by phytochemicals, including the potential for mediating antiestrogenic or estrogenic responses by mechanisms independent of direct binding to human ERs. This discovery suggests that possible alternate signaling pathways may regulate ER function in an antagonistic manner. Further evaluation of the cell type-specific effects and potential regulation of other steroid hormone receptors by these chemicals may elucidate unique hormone receptor antagonistic signaling pathways. The determination and elucidation of a negative regulatory pathway for estrogens may have profound implications by facilitating new targets for the design of novel antiestrogens and determining how the daily exposure to phytochemicals may impact human health (environmental relevance).

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Phytochemical Glyceollins, Isolated from Soy, Mediate Antihormonal Effects through Estrogen Receptor α and β*

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ABSTRACT

The flavonoid family of phytochemicals, particularly those derived from soy, has received attention regarding their estrogenic activity as well as their effects on human health and disease. In addition to these flavonoids other phytochemicals, including phytostilbene, enterolactone, and lignans, possess endocrine activity. The types and amounts of these compounds in soy and other plants are controlled by both constitutive expression and stress-induced biosynthesis. The health benefits of soy-based foods may, therefore, be dependent upon the amounts of the various hormonally active phytochemicals within these foods. The aim was to identify unique soy phytochemicals that had not been previously assessed for estrogenic or antiestrogenic activity. Here we describe increased biosynthesis of the isoflavonoid phytoalexin compounds, glyceollins, in soy plants grown under stressed conditions. In contrast to the observed estrogenic effects of coumestrol, daidzein, and genistein, we observed a marked antiestrogenic effect of glyceollins on ER signaling, which correlated with a comparable suppression of 17β-estradiol-induced proliferation in MCF-7 cells. Further evaluation revealed greater antagonism toward ERα than ERβ in transiently transfected HEK 293 cells. Competition binding assays revealed a greater affinity of glyceollins for ERα *vs.* ERβ, which correlated to greater suppression of ERα signaling with higher concentrations of glyceollins. In conclusion, we describe the phytoalexin compounds known as glyceollins, which exhibit unique antagonistic effects on ER in both HEK 293 and MCF-7 cells. The glyceollins as well as other phytoalexin compounds may represent an important component of the health effects of soy-based foods. (*J Clin Endocrinol Metab* **86:** 1750–1758, 2001)

FLAVONOIDS REPRESENT a family of phytochemicals that function to deter herbivores, act as antibacterial/antifungal agents, and stimulate the formation of symbiotic relationships with nitrogen-fixing bacteria (1–3). The family of flavonoids is often subclassified into groups of chemicals referred to as flavones, isoflavonoids, chalcones, and coumestans based on their shared structural similarity. Although the functions of these diverse compounds are not completely understood, they not only affect bacteria and fungi, but have been reported to exert effects on mammals as well (1–5). The observations of sheep grazing on fields rich in clover and cheetahs fed high soy diets in zoos have demonstrated that flavonoids and related phytochemicals can affect mammalian health (5–7). Of interest was the observation that these compounds function as estrogenic mimics or phytoestrogens and may represent important dietary factors affecting human health (8–13). The estrogenic phytochemicals, which include flavonoids, lignans, phytostilbenes, and enterolactones, appear to primarily function by binding to and activating the estrogen receptor (ER), albeit at 100-1000 greater concentrations than 17β-estradiol (14–18). Two key constitutive isoflavonoids most often detected in soybean tissue, genistein and daidzein, have been widely examined for these effects. The observation that soy phytochemicals can function as estradiol (E_2) agonists is consistent with the observed health benefits of soy foods, such as decreased incidence of osteoporosis and cardiovascular disease (8–13, 19–23). However, the similar decrease in risk of breast cancer would indicate a potential antiestrogenic activity of soy phytochemicals (17–21). Additionally, the ability of soy isoflavonoids to prevent carcinogen-induced mammary tumorigenesis further demonstrates the potential antiestrogenic effects of these compounds. Consistent with this information, certain phytochemicals have been reported to exert anties-

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trogenic effects at higher concentrations (17, 18). These studies, however, were not exclusive to soy-derived isoflavonoids, suggesting that many flavonoids may function as both ER agonists and antagonists in a dose- and cell type-specific manner. The recent identification of a second estrogen receptor β (ERβ) with different affinity for and *trans*-activation by phytoestrogens represents another mechanism by which flavonoids may function to regulate estrogen signaling (24–26).

The suggestion that the high isoflavonoid content of soy may function to prevent cancer and disease is bolstered by the observation that the predominant isoflavonoids found in soy, genistein and daidzein, can affect estrogen signaling and prevent cancer in animal models. However, genistein and daidzein represent only two compounds in the complex flavonoid biosynthetic pathway, as shown in Fig. 1A, and the amount and type of isoflavonoid present in soy can be readily altered in response to external stimuli. The recent demonstration that the soy isoflavonoid glycitein can function as an estrogen illustrates that other isoflavonoids must be considered in relation to the health effects of soy products (27). Additionally, environmental factors and growth conditions can alter the biosynthesis leading to the production of numerous flavonoids that have not been characterized for their effects in mammalian systems (28–30).

Phytoalexins constitute a chemically heterogeneous group of substances belonging to the various subclassifications of flavonoids mentioned above. Phytoalexins are low molecular weight antimicrobial compounds that are synthesized *de novo* and accumulate in plants as a stress response (4, 31). The phytoalexins are generally lipophilic compounds that are products of a plant's secondary metabolism and often accumulate at infection sites at concentrations that inhibit fungal and bacterial growth (4, 31). Countless stress factors or physical stimuli induce phytoalexin accumulation, including freezing, UV light exposure, and exposure to microorganisms. In addition, compounds referred to as elicitors, either abiotic or biotic, can stimulate the biosynthesis of phytoalexins (4, 28–29, 31–36). Given that the biosynthesis of isoflavonoids, particularly phytoalexins, can be regulated by external factors, the type and amount of hormonally active phytochemicals may vary from source to source. Additionally, the environmentally induced biosynthesis of unique isoflavonoids of undefined hormonal activity may represent an important component of both the beneficial and/or detrimental effects of these compounds on human health.

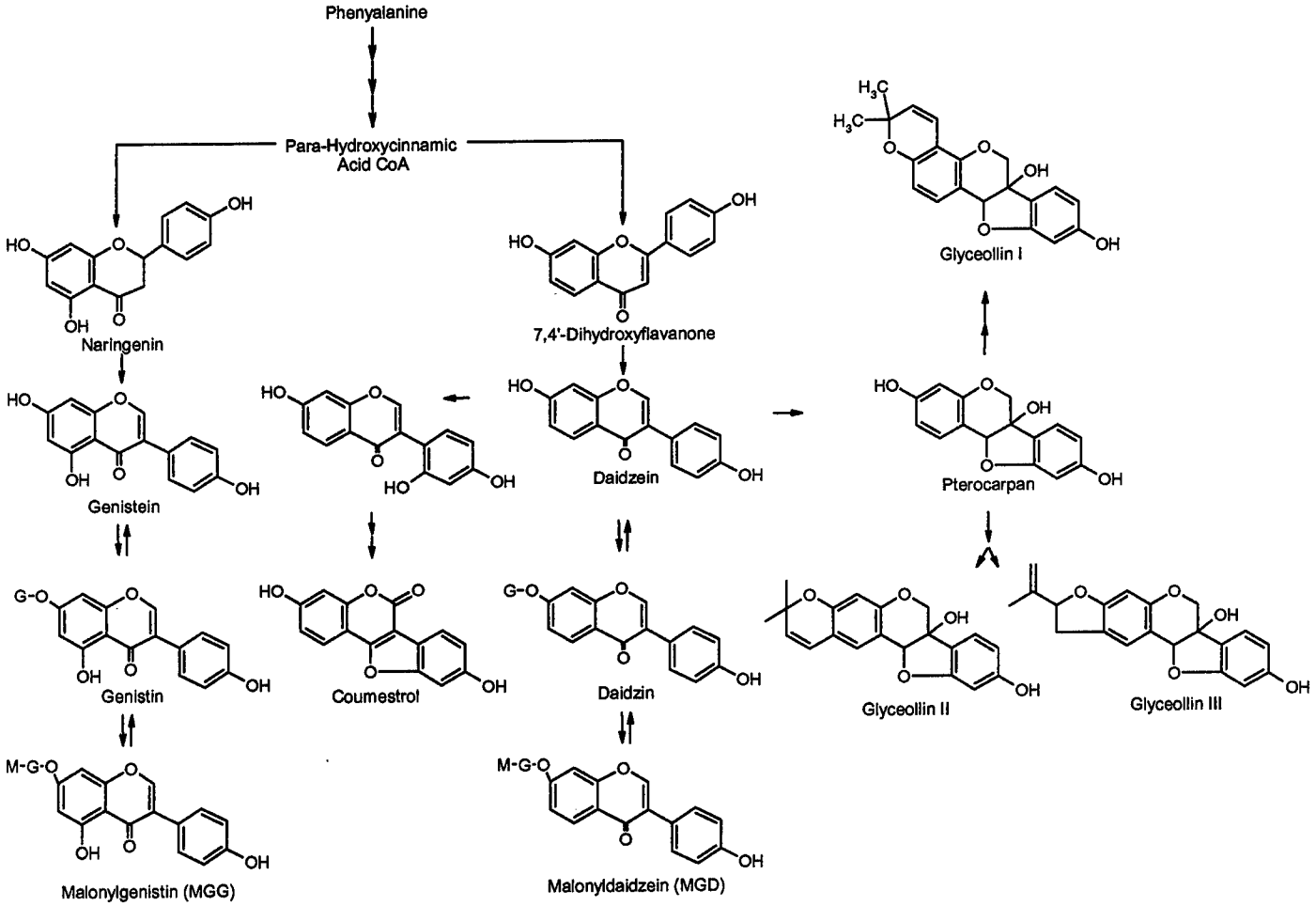

FIG. 1. Flavonoid pathway showing the biosynthetic route from phenylalanine to coumestrol, genistein, daidzein, and the conjugated forms, daidzin, genistin, MGD, and MGG. Also detailed is the biosynthetic pathway from daidzein to glyceollins I–III.

The specific aim of this study was to identify unique soy phytochemicals that have not been previously assessed for estrogenic or antiestrogenic activity and determine whether the altered biosynthesis of flavonoids represents a point of regulation of the hormonal activity of soy products. The present study describes induction of the soybean phytoalexins glyceollins I–III by the fungus *Aspergillus sojae*, a non-toxin-producing *Aspergillus* strain commonly used in the fermentation of soybeans to produce soy sauce and miso. The glyceollins represent a group of phytoalexins whose biosynthesis is increased in response to stress signals. The glyceollin isomers I–III have core structures similar to that of coumestrol and are derived from the precursor daidzein in the glyceollin pathway (see Fig. 1B). The ability of the glyceollins to regulate estrogen signaling was analyzed using the ER-positive MCF-7 human breast carcinoma cell line and ER-negative HEK 293 cells transfected with either ERα or ERβ. Although the glyceollins displayed only slight estrogenic activity, they did cause a dose-dependent suppression of 17β-estradiol-induced *trans*-activation and MCF-7 cell proliferation. The glyceollins also functioned to suppress estrogen activity through both ERα and ERβ, which correlated with binding to ERα and ERβ, respectively. Here we describe the isoflavonoid phytoalexins known as glyceollins I–III, which are synthesized in soy under stress conditions and exhibit a unique antagonistic effect on ER activity in a number of hormone-responsive systems.

Materials and Methods

Chemicals and plasmids

The isoflavonoids daidzein, genistein, and coumestrol were obtained from Indofine Chemical Co. (Somerville, NJ). 4-Hydroxytamoxifen was purchased from Sigma (St. Louis, MO). ICI 182,780 was provided by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Glyceollins I, II, and III were isolated using a procedure developed in this laboratory. Soybean seeds (50 g) were sliced and inoculated with *Aspergillus sojae*. After 3 days isoflavonoids were extracted from the inoculated seeds with 80% ethanol. The glyceollins were isolated using preparative scale high pressure liquid chromatography (HPLC) and were confirmed by UV-visable spectrophotometry and electrospray mass spectrometry. A mixture of glyceollins I, II, and III in a ratio of 6:2:1 was isolated and used in subsequent analyses. The solvents acetonitrile (HPLC grade) and ethanol were purchased from Aldrich Chemical Co., Inc. (Metuchen, NJ). H_2O treated with a Millipore Corp. system (Bedford, MA) was used during sample preparation procedures and HPLC analyses.

ERβ complementary DNA (cDNA) was provided by Jan-Åke Gustafsson (Karolinska Institute, Stockholm, Sweden) in pBluescript. ERα and ERβ expression vectors were constructed by inserting the ERα and ERβ cDNA, respectively, into pcDNA 3.1 vector (Invitrogen, San Diego, CA). ERα cDNA (2090 bp) was cleaved from plasmid (pBluescript) with *Bam*HI/*Eco*RI and then ligated into the pcDNA3.1. ERβ cDNA (1460 bp) was cleaved from Plasmid (pBluescript) with *Hin*dIII/*Bam*HI and then ligated into the pcDNA3.1. Each construct was verified by detailed restriction mapping.

Soybean treatment and harvesting

A. sojae (SRRC 1125) cultures were grown at 25 C in the dark on potato dextrose agar. After 5 days inoculum was prepared by harvesting conidia (3.4×10^7/mL) in 15 mL sterile distilled H_2O. Buckshot 66 soybean was donated by Louisiana State University Agricultural Center (Baton Rouge, LA). Seeds from commercial soybean variety Buckshot 66 were surface-sterilized for 3 min in 70% ethanol, followed by a quick deionized H_2O rinse and two 2-min rinses in deionized H_2O. Seeds were presoaked in sterile deionized H_2O for 4–5 h before placement into treatment chambers (three seeds per chamber). Each chamber consisted of a petri dish (100 × 15 mm, four compartments); each compartment was lined with two autoclaved filter papers (Whatman, Clifton, NJ) moistened with 0.5 mL distilled H_2O. One seed was placed into a single compartment then sliced in half longitudinally. *A. sojae* spore suspension (10 μL) was applied to the cut surface of each seed. All chambers were stored at 25 C in the dark for 3 days, then transferred to −70 C. Soy extracts were prepared from both *A. sojae*-inoculated and noninoculated 3-day-old seeds. Soy extracts were extracted from 5 g finely ground seeds in 8 mL ethanol and heated at 50 C for 1 h, cooled, then centrifuged at 14,000 × *g* for 10 min. Extracts were filtered through 0.45-μm pore size sterile filter units (Gelman Sciences, Ann Arbor, MI). Stock solutions were prepared as follows. Two milliliters of each extract were evaporated to dryness and dissolved in dimethylsulfoxide at a concentration of 100 mg/mL.

HPLC analyses of phytochemicals

HPLC analyses were performed on a Waters 600E System Controller combined with a Waters UV-visable 486 detector (Waters Corp., Milford, MA). Soy isoflavonoids were extracted from cotyledons (0.3–0.6 g) and homogenized (Tekmar Tissumizer; Tekmar Co., Cincinnati, OH) in 1.5 mL 80% ethanol. Homogenate was heated at 50 C for 1 h, cooled, then centrifuged at 14,000 × *g* for 10 min, and the supernatant was run on HPLC. An aliquot (100 μL) of supernatant was directly analyzed by HPLC. Isoflavonoids were monitored at a wavelength of 260 nm, but the glyceollins were monitored at 285 nm. Separations were carried out using a Multiring C_{18} (4.6 × 250 mm; 5 μm; Vydac, Hesperia, CA) reverse phase column. A guard column containing the same packing was used to protect the analytical column. Elution was carried out at a flow rate of 1.0 mL/min with the following solvent system: A = acetic acid/water (pH 3.0); B = acetonitrile; 0% B to 45% B in 17 min, then 45% B to 90% B in 10 min followed by holding at 90% B for 6 min. Retention times for the isoflavonoids were as follows: daidzin (13.4 min), genistin (15.0 min), malonyldaidzin (MGD; 15.3 min), malonylgenistin (MGG; 16.7 min), daidzein (17.8 min), genistein (20.1 min), coumestrol (20.7 min), glyceollin III (23.3 min), glyceollin II (23.6 min), and glyceollin I (23.7 min). Calibration curves with high linearity were constructed for each isoflavonoid using a series of diluted standards (daidzin and genistin were used for MGD and MGG, respectively). All HPLC analyses were run in triplicate unless otherwise stated.

Cell culture

MCF-7 cells and human embryonic kidney (HEK) 293 cells were cultured in 150-cm^2 culture flasks in DMEM supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), basic minimum essential and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (diluted in the medium to a 1-fold concentration from either 100- or 50-fold stocks), and porcine insulin (10^{-8} mol/L; Sigma). The culture flasks were maintained in a cell incubator in a humidified atmosphere of 5% CO_2 and 95% air at 37 C. The MCF-7 cells used here (N variant) express predominantly ERα, with weak expression of ERβ, as previously described (36a).

Luciferase assays

As previously described (37, 38), MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 48 h before plating. The cells were plated in 12-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 1 μg pGl2-ERE2X-TK-luciferase plasmid [containing two copies of the vitellogenin estrogen response element (ERE) linked to the luciferase gene; TK, tyrosine kinase] using 3 μL Lipofectamine (Life Technologies, Inc.)/μg DNA. HEK 293 cells were plated in 12-well plates at 5×10^5 cells/well in 5% CS-FBS, allowed to attach overnight, then transfected with 1 μg pGl2-ERE2X-TK-luciferase plasmid and either 500 ng pcDNA3.1B-ERα or 10 ng pcDNA3.1B-ERβ. After 5 h the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% CS-FBS containing vehicle, 17β-estradiol, phytochemical, or 17β-estradiol plus phytochemical and incubated at 37 C. After 18 h the medium was removed, and 200 μL 1 × lysis buffer (Promega Corp., Madison, WI) were added per well and

incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15,000 × *g* for 5 min. The cell extracts were normalized for protein concentration using reagent following the protocol supplied by the manufacturer (Bio-Rad Laboratories, Inc., Hercules, CA). Luciferase activity for the cell extracts were determined using luciferase substrate (Promega Corp.) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

MCF-7 cell proliferation assay

The MCF-7 cell proliferation assay used is a modified version of published methods (39–41). MCF-7 cells were placed in phenol red-free DMEM supplemented with 10% 5% CS-FBS 7 days before plating. The cells were plated in 96-well plates at 4.5 × 10^3 cells/well (~10% confluence) in 100 μL of the same medium. After 24 h the cells were dosed with treatment medium at 100 μL/well. Treatment medium consisted of 10% dextran-coated charcoal-FBS into which phytochemicals and controls in ethanol carrier were added (0.1% ethanol, vol/vol). The experimental cells were retreated with phytochemicals on day 4. Cell proliferation was measured on day 7 when positive control wells reached 90–100% confluence. Alamar Blue dye was added to the medium (10 μL/well), and the plates were incubated for 3 h at 37 C with 5% CO_2. Fluorescence was monitored at 560 nm excitation and 590 nm emission using a FluoroLite 1000 (Dynatech Corp., Chantilly, VA). Within proliferation assays, each dose was run in four wells. Reported data are the mean (±SD) of three independent experiments.

ERα and ERβ binding analysis

The ERα and ERβ binding assays were performed using a modification of previously reported methods (42). A PanVera CoreHTS ER kit was used for both ERα and ERβ experiments. A 26-nmol/L ERα solution was added to a fluorescent estrogen (2 nmol/L Fluormone ES2; Panvera, Madison, WI) ligand to form an ES2/ERα complex with high fluorescence polarization. Fifty microliters of the ES2/ERα complex were added to sample tubes containing 50-μL serial dilutions of test phytochemicals and were mixed well by shaking. A control tube containing 50 μL ES2 screening buffer and 50 μL ES2/ERα complex was used as a negative control to determine the polarization value with no competitor present and represented 0% competition. E_2 was used as a control on each plate. The tubes were incubated in the dark at room temperature (22 C) for 2 h. Polarization values were read using a Becon 2000 fluorescence polarization instrument (Panvera) at 485 nm excitation and 530 nm emission. Each data point in the proliferation assay was run in triplicate, and reported data are the mean (±SD) of three experiments.

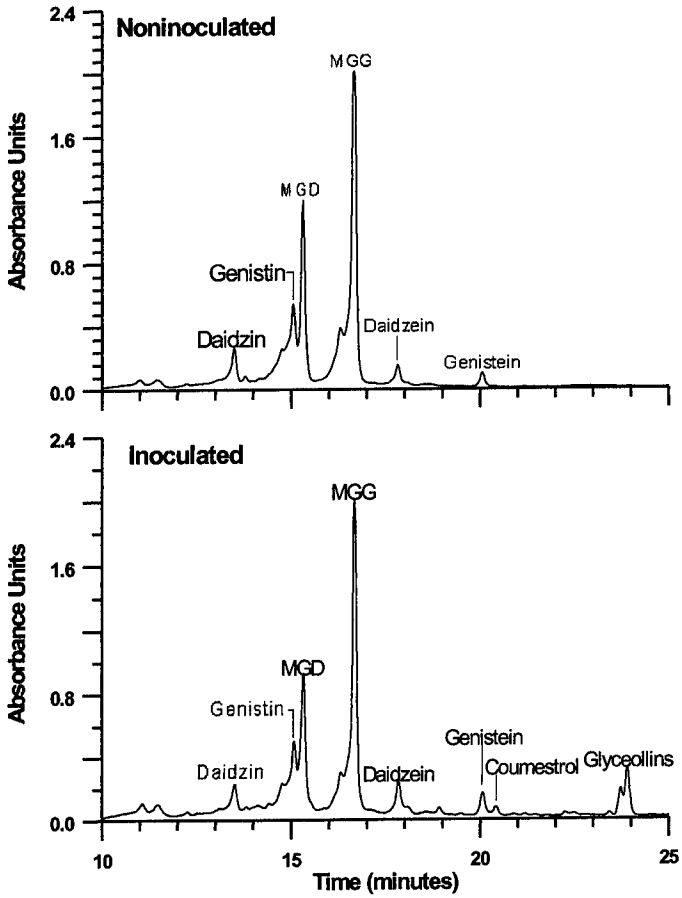

FIG. 2. Elicitor-mediated alteration of the soybean isoflavonoid profile. HPLC comparison between noninoculated and inoculated soybean cotyledons. A, HPLC chromatogram of 3-day-old noninoculated cotyledons, showing constitutive isoflavonoids; B, HPLC chromatogram of 3-day-old cotyledons inoculated with *A. sojae*, detailing the induction of coumestrol and glyceollin isomers I, II, and III. The data represent steady state amounts of glyceollins I–III and coumestrol at or near their peak levels after 3 days at 260 nm.

Results

Changes in isoflavonoid levels in cotyledons inoculated with *A. sojae* were analyzed using HPLC. A representative HPLC profile comparison between noninoculated and inoculated soybean cotyledons with *A. sojae* is displayed in Fig. 2. Figure 2A displays the HPLC chromatogram obtained from noninoculated cotyledon tissue. The more prevalent constitutive isoflavonoids, daidzin, genistin, MGD, MGG, daidzein, and genistein, are present. The HPLC assay used in this study did not detect trace levels of glyceollin in the noninoculated soybean cotyledon tissue. Figure 2B displays the HPLC chromatogram obtained from *A. sojae*-inoculated cotyledon tissue. The induction of high concentrations (1117 μg/g) of the glyceollin isomers I–III is clearly shown. This concentration of total glyceollin is relatively high compared with the concentrations of daidzein and genistein, and experiments conducted in our laboratory have indicated that glyceollin can represent up to 56% of the total isoflavonoid composition of the inoculated soybean cotyledon (see Table 1). Low levels of coumestrol were detected in inoculated soybean cotyledons (30 μg/g).

The reported estrogenic effects of soy and soy foods are primarily due to the soy isoflavonoids genistein, daidzein, and glycitein. These isoflavonoids as well as coumestrol and other flavonoids predominantly act as estrogenic chemicals, but also exhibit antiestrogenic activity in a dose-dependent manner (17, 18, 43). Therefore, both the specific type and amount of flavonoids present will determine the overall estrogenic activity. Based upon the observed differences in isoflavone profiles in normal *vs.* elicited soy, extracts were used to examine the overall estrogenic activity of soy under these two conditions. Using a estrogen-responsive reporter gene assay in MCF-7 human breast carcinoma cells we observed a difference in relative estrogenic activity between these two extracts (Fig. 3A). Although normal soy extract resulted in a maximal 94% estrogenic activity occurring at 100 μg/mL, a maximal 69% activity was observed with treated soy extract (100 μg/mL). Similar experiments were performed using elicited or normal soy extracts in combination with 17β-estradiol treatment to assess antiestrogenic activity (Fig. 3B). Normal soy extract did not exhibit antiestrogenic activity at any concentration tested, with activity in combined treatments remaining at or above that with estro-

TABLE 1. Phytoestrogen composition of noninoculated and *A. sojae*-inoculated soybean seeds

Phytoestrogen	Noninoculated soybean seed (μg/g dry wt)	*A. sojae*-inoculated soybean seed (μg/g dry wt)
Daidzein	7 ± 0.5	37 ± 10
Genistein	11 ± 1	20 ± 7
Daidzin	199 ± 40	93 ± 6
Genistin	249 ± 15	119 ± 29
Malonyldaidzin	380 ± 33	239 ± 43
Malonylgenistin	468 ± 10	344 ± 33
Coumestrol	N/D	20 ± 6
Glyceollins[a]	N/D	1117 ± 229

Results are the averages of triplicate experiments. N/D, Not detected.

[a] Total of glyceollins I, II, and III.

gen alone (100%). In contrast, the elicited soy extract decreased estrogen's activity below 100% at concentrations of 1–100 μg/mL, with a maximal decrease to 55% at 100 μg/mL. Therefore, the relative difference in isoflavone content between treated and untreated soy observed by HPLC analysis correlated with a decreased estrogenic profile in the treated soy extracts. Interestingly, this decreased estrogenic activity occurred despite increased levels of coumestrol, glyceollins I–III. Alone, coumestrol is a potent estrogenic compound, whereas glyceollins appear nonestrogenic (Fig. 4A). This suggested that the altered profile of flavonoids, particularly the presence of novel chemicals (*i.e.* glyceollins) might be responsible for the observed antiestrogenic effects of elicited soy. We next investigated the effects of isolated soy isoflavonoids on estrogenic signaling. Consistent with previous results, genistein, coumestrol, and daidzein demonstrated a dose-dependent activation of the estrogen response in MCF-7 cells (Fig. 4A), with coumestrol showing the greatest activity (90% at 100 nmol/L), followed by genistein (110% at 1 μmol/L) and daidzein (150% at 10 μmol/L). Treatment with the glyceollins from 10 nmol/L to 25 μmol/L displayed only weak activity at 10 nmol/L equivalent to 25% of that of E_2 (1 nmol/L; Fig. 4A). To determine whether the glyceollins acted as antiestrogens, MCF-7 cells were transfected with ERE-luciferase and treated with E_2 in addition to increasing concentrations of glyceollins (10 nmol/L to 25 μmol/L). As shown in Fig. 4B, these assays revealed that despite the lack of agonistic activity, the glyceollins demonstrated antagonistic activity in MCF-7 cells between 1 and 25 μmol/L concentrations. The antiestrogenic activity of glyceollins as observed in breast carcinoma cells was further evaluated using ER-positive Ishikawa human endometrial carcinoma cells. Although little or no agonistic activity was observed in these cells, glyceollins did display antagonistic activity, but at higher concentrations than observed in MCF-7 cells (data not shown).

The proliferation of MCF-7 cells is a well established biological response to 17β-estradiol and a useful screening tool for compounds that may function as estrogen agonists (38–40). Additionally, E_2-induced proliferation can be blocked by the addition of antiestrogenic compounds such as ICI 182,780 or tamoxifen. Here we demonstrate that estrogen alone is capable of stimulating MCF-7 cell proliferation (3.6 ± 1.2-fold), as measured using an Alamar Blue staining technique

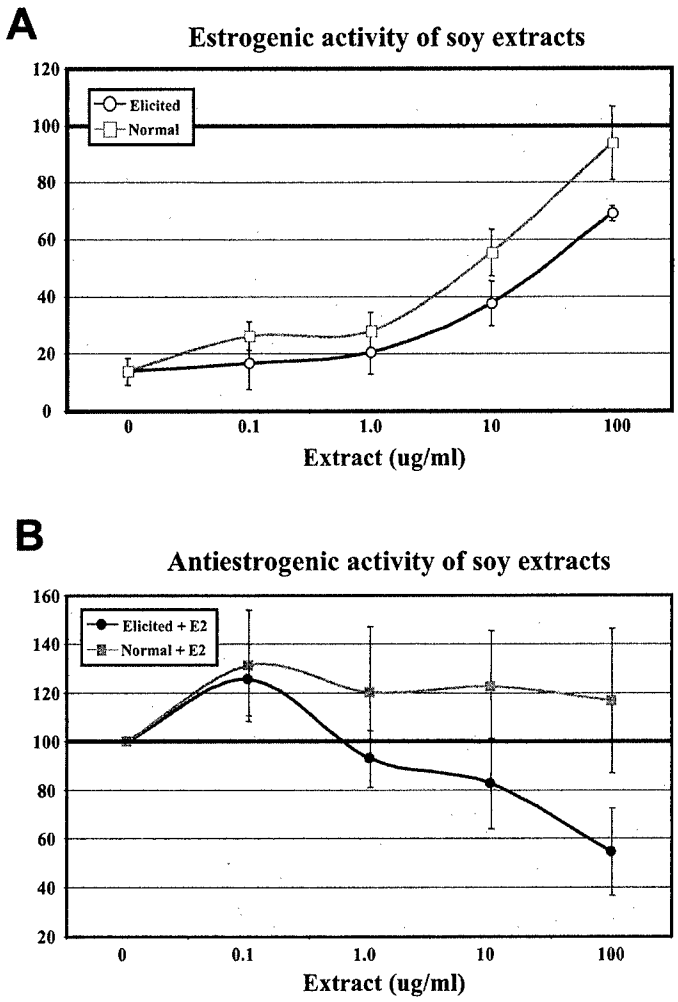

FIG. 3. Estrogenic and antiestrogenic effects of normal *vs.* elicited soy extracts. MCF-7 cells were transfected with an ERE-Luc plasmid for 6 h, treated overnight, and harvested for luciferase activity. Cells were treated with increasing doses (1–100 μg/mL) of normal (●) or elicited (●) soy extracts (A) or were treated with E_2 (1 nmol/L) in combination with normal (●) or elicited (●) soy extracts (B). Data are represented as the percent estrogenic activity as determined from 1 nmol/L E_2 alone (100%) ± SEM of three experiments.

(Fig 5). The addition of 100 nmol/L ICI 182,780 inhibited E_2-stimulated proliferation (data not shown), whereas treatment with 100 nmol/L ICI 182,780 alone maintained cell proliferation at levels similar to those after treatment with medium and carrier solvent alone (−5.2 ± 1.3%). The glyceollins alone showed a low level of estrogenic activity; however, at 10 μmol/L the estrogenic activity increased to 62%. The dose-dependent addition of the glyceollins suppressed the E_2-stimulated proliferation (100%) to 71% and 30% at 10 and 25 μmol/L, respectively. Interestingly, the glyceollins alone at 10 μmol/L were capable of increasing proliferation to 62%. However, ICI 182,780 was unable to block this proliferation, suggesting that an alternate, non-ER-related signaling pathway was involved.

There has been significant recent interest in the newly identified ERβ (25, 26). Previous studies have demonstrated some differences in ligand binding specificity and *trans*-activation between the α and β ERs (18, 26, 44, 45). Of par-

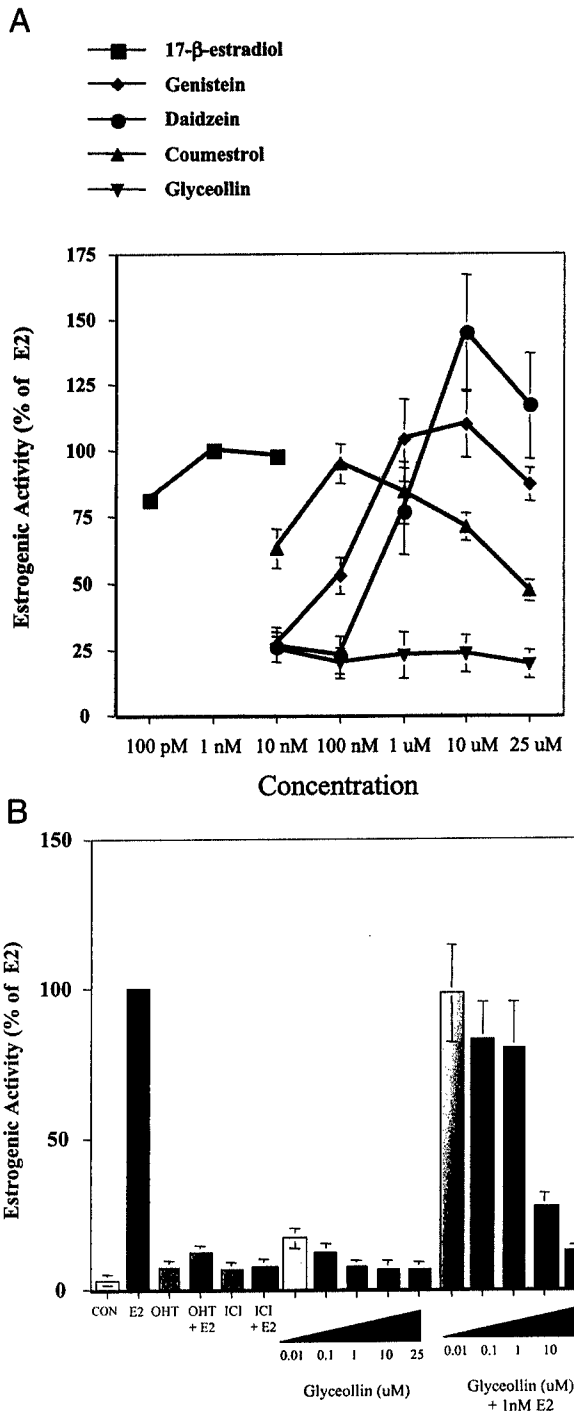

FIG. 4. Estrogenic and antiestrogenic activities of glyceollin in MCF-7 breast carcinoma cells. MCF-7 cells were transfected with an ERE-luciferase plasmid for 6 h, treated, and harvested for luciferase activity the following day. Data are presented as the percent estrogenic activity relative to 1 nmol/L E_2 (●; 100%). A, Estrogenic activity of the isoflavones daidzein (●), genistein (▲), and coumestrol (●) and the phytoalexin glyceollin (●) determined by treatment with increasing concentrations (10 nmol/L to 25 μmol/L) of phytochemical. B, Antiestrogenic activity was determined using glyceollin (10 nmol/L to 25 μmol/L) in combination with 1 nmol/L E_2. The antiestrogenic effects of glyceollin were compared with those of 100 nmol/L 4-hydroxytamoxifen (OHT) and 100 nmol/L ICI 182,780 (ICI) alone or in combination with 1 nmol/L E_2. *Data points* and *error bars* represent the mean ± SEM of three experiments per each concentration tested.

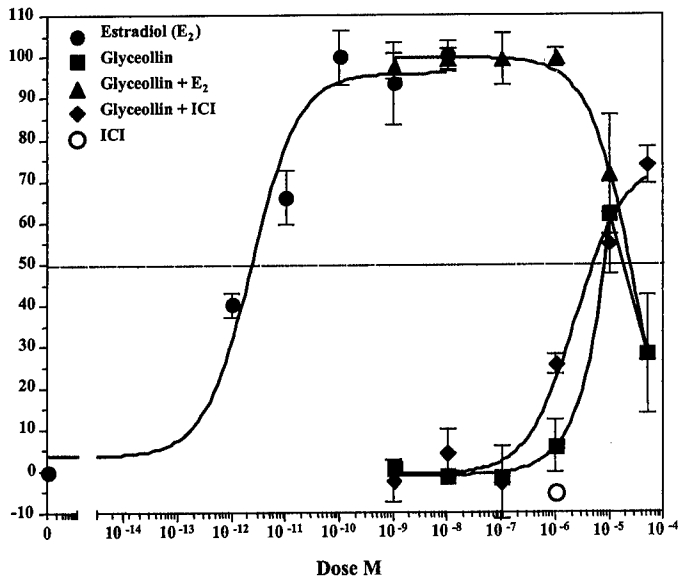

FIG. 5. Estrogenic and antiestrogenic activities of glyceollin at varying concentrations using an MCF-7 cell proliferation assay. Cell proliferation was determined using an Alamar Blue assay and is expressed relative to E_2 (100%) at 0.1 nmol/L (●). The proliferative effects of glyceollin (1 nmol/L to 50 μmol/L) are shown alone (●), in combination with 10 nmol/L E_2 (●), or in combination with 1000 nmol/L ICI 182,780 (▲). *Data points* and *error bars* represent the mean ± SD of three experiments.

ticular interest was the observation that certain flavonoid phytochemicals may bind with higher affinity and possess higher agonistic action toward ERβ (25, 26, 44–48). To assess the ability of the glyceollins to bind to ERα and ERβ, a competitive binding assay with fluorescent detection was used. Figure 6A details the results for the competitive binding assay using ERα. A displacement to 50% ES2 bound to ERα occurred at a concentration of 5 nmol/L. The IC_{50} of the glyceollins for ERα was 3.2 μmol/L. However, as shown in Fig. 6B, the IC_{50} of the glyceollins for ERβ was 6.4 μmol/L. This indicated that the ability of glyceollin to act as an ER antagonist occurred through receptor binding, and the greater affinity for ERα *vs.* ERβ correlated with the preferential antagonism of ERα activity.

To determine whether the glyceollins exhibit higher activity toward either receptor, transient transfection was performed using the ER and ER-negative cell line HEK 293. Cotransfection of either ERα or ERβ along with an ERE-luciferase construct allowed examination of the effects of receptor-specific estrogenic or antiestrogenic effects of glyceollin. Treatment with 17β-estradiol resulted in 14- and 8.4-fold *trans*-activation comparable to controls of ERα and ERβ, respectively. These results are consistent with the observations that MCF-7 cells treated with glyceollins from concentrations of 100 nmol/L to 25 μmol/L did not significantly activate an ERE response. However, ERα-transfected cells treated with 1 nmol/L E_2 at 100%, when combined with glyceollins, produced a dose-dependent decrease in ER activity to 42% and 15% at 10 and 25 μmol/L (see Fig. 7A). Using ERβ-transfected cells glyceollin was capable of suppressing β signaling to 60% and 45% of E_2 at similar glyceollin concentrations (Fig. 7B). Both the synthetic estrogen

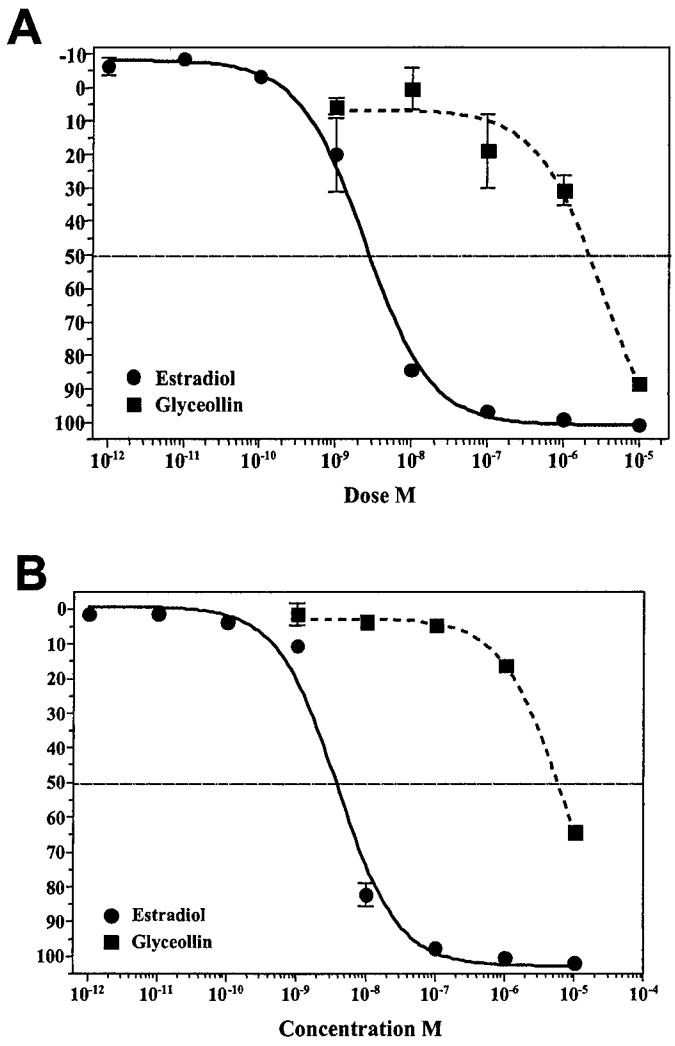

FIG. 6. Competition binding curves of glyceollin and ER (ERα and ERβ). Increasing concentrations of glyceollin (1–10 μmol/L; ●) were added to ERα/ES2 complex (A) and ERβ/ES2 complex (B) and compared with E_2 (●). *Data points* and *error bars* represent the mean ± SD of three experiments (n = 3) for each concentration tested.

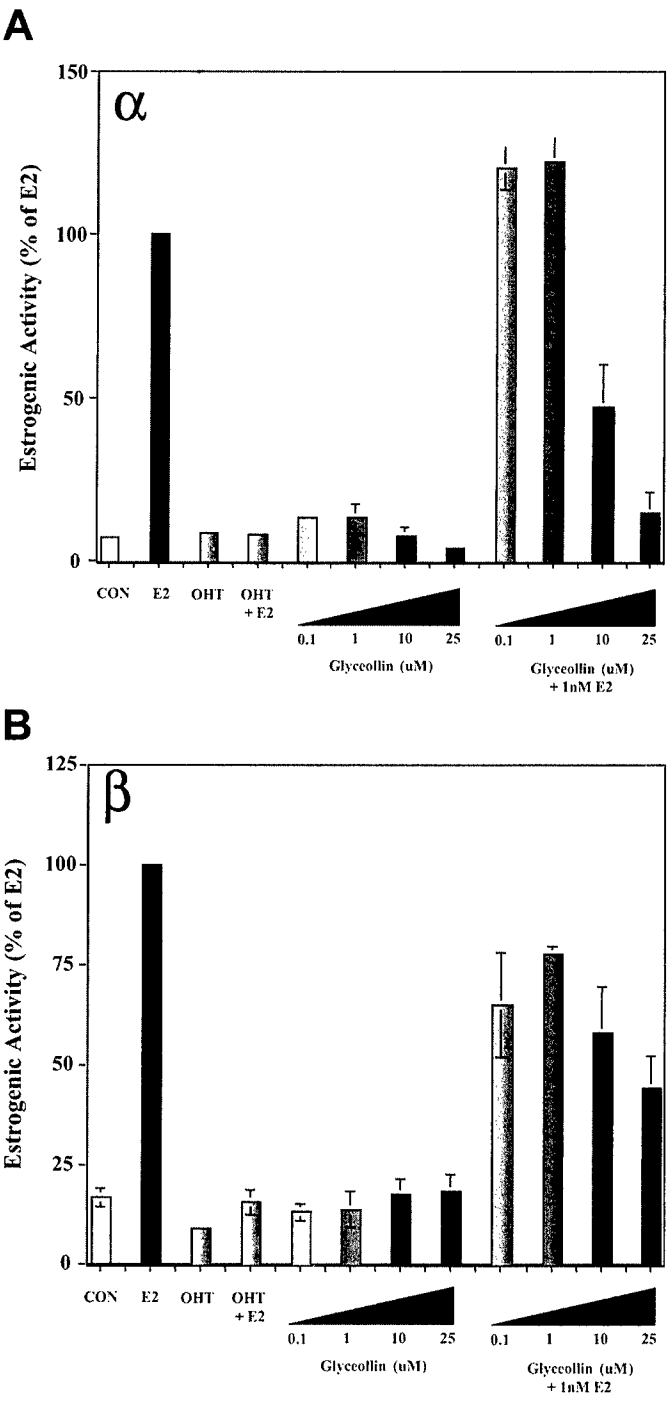

FIG. 7. ERα- and ERβ-specific effects of glyceollin. The estrogenic and antiestrogenic activities of glyceollin (0.1–25 μmol/L) were examined using HEK293 cells transfected with an ERE luciferase plasmid along with either ERα (A) or ERβ expression vectors (B), with E_2 at 1 nmol/L representing 100% activity. The antagonistic activity of glyceollin on ERα and ERβ was examined alone or in combination with 1 nmol/L E_2. *Data points* and *error bars* represent the mean ± SEM of four experiments for each concentration tested.

diethylstilbestrol (DES; 1 nmol/L) and the phytoestrogen genistein (1 μmol/L) have been shown to function as estrogens in ERα- or ERβ-transfected HEK 293 cells. Consistent with these studies, DES (1 nmol/L) and genistein (1 μmol/L) both stimulated ERE-Luc activity to a similar extent as E_2 (1 nmol/L; data not shown). The antiestrogenic effect of the glyceollins was examined using DES or genistein as an activator of ERE-luciferase. The glyceollins displayed similar preferential suppression of ERα signaling compared with ERβ activated by either DES or genistein.

Discussion

Given the significant interest in the estrogenic activity of isoflavonoids, this study was undertaken to determine the hormonal activity of the isoflavonoid phytoalexin glyceollin. Glyceollin accumulates in high concentrations in soybeans under conditions of stress, and little is know about its hormonal effects in mammalian systems. The presence of glyceollin and other phytoalexins in foods obtained from stressed plants presents a potential hazard to human health. The HPLC profiles in Fig. 2 demonstrate that glyceollin is readily extracted with daidzein and genistein and can represent as much as 56% of the total isoflavone composition. The availability of daidzein and genistein in processed soy

foods, including soy protein (49, 50), leads to the conclusion that glyceollin would be present along with the other constitutive isoflavones in soy foods prepared from treated (stressed) soy. Therefore, the glyceollins were examined in a variety of hormone-responsive systems and, in contrast to previously identified soy isoflavonoids, demonstrated antiestrogenic effects in these systems. Studies with MCF-7 cells revealed the glyceollins suppressed both E_2-mediated gene *trans*-activation and E_2-mediated proliferation when applied at similar concentrations. However, the glyceollins alone were capable of only slightly enhancing MCF-7 cell proliferation. This effect was not suppressed by combination with the antiestrogen ICI 182,780, suggesting an ER-independent mechanism. Several flavonoids have been demonstrated to influence effects on other signaling pathways, such as tyrosine kinases, mitogen-activated kinases, and protein kinase C inhibition (3, 46–48). The ability of the glyceollins to induce proliferation may therefore be mediated through an unrelated pathway. Additionally, we have shown that that certain flavonoids, unable to compete for ER binding, inhibited both E_2-mediated gene expression and proliferation, potentially through undefined alternate signaling pathways. To confirm that the antiestrogenic effects of the glyceollins occurred through direct receptor interaction, binding analyses of the glyceollins with both ERα and ERβ were performed. These studies showed that the glyceollins demonstrated a slightly greater affinity for ERα than for ERβ. The antiestrogenic activity observed in MCF-7 was further evaluated using ER-negative HEK 293 cells transfected with either ERα or ERβ. These studies demonstrated that the glyceollins suppressed E_2-induced *trans*-activation through ERα to a greater extent than ERβ. Similar results were obtained using either a known estrogenic isoflavonoid genistein or the synthetic estrogen DES. Previous reports (25, 26, 44, 45) demonstrated greater binding to and activation of ERβ *vs.* ERα by phytoestrogens. In contrast to these reports, the antiestrogenic effects of the glyceollins appear to be due to the greater affinity toward ERα.

Significant research has previously identified a potential role for soy and soy foods in the prevention of human disease and the promotion of health. These effects, including decreased risk of certain types of cancers as well as prevention of cardiovascular disease and osteoporosis, have been linked to the estrogenic isoflavonoids genistein and daidzein present in soy. However, the relative amounts of these two isoflavonoids and the glucose-conjugated forms vary dramatically among soybean varieties (28, 30) and the type of soy food prepared (49, 50). Additionally, daidzein and genistein are not the only isoflavonoids found in soybeans. The recent report by Song *et al.* demonstrated the estrogenic activity of glycitein, an isoflavonoid also detected in both soy and soy foods (27). Extensive work has shown that the amount and type of isoflavonoids found within legumes are dependent upon plant growth conditions, and that biosynthesis of these compounds can be significantly altered under conditions of stress (4, 28, 29, 31–36). The type and amount of these compounds may influence the overall estrogenic activity of soy-based foods. We have demonstrated both estrogenic and antiestrogenic effects of numerous other flavonoid compounds (17, 18), suggesting that isoflavonoids besides genistein and daidzein may be important in the health benefits of these compounds. Recent studies have also demonstrated that flavonoids from red clover and hops (23, 51) possess estrogenic effects and may represent important considerations in human health. Here we describe the phytochemical isoflavonoid glyceollin as being induced in soybean plants grown under conditions of stress. The lack of agonistic activity of the glyceollins in combination with weak, but significant, antiestrogenic activity are of interest. In contrast to the observed estrogenic effects of many soy isoflavonoids and other flavonoids, the antiestrogenic effects of glyceollins may also be considered important with regard to their presence in soy-based foods.

Acknowledgments

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Erratum

In the article "Degree of fatness after treatment for acute lymphoblastic leukemia in childhood" by Karsten Nysom, Kirsten Holm, Kim Fleischer Michaelsen, Henrik Hertz, Jørn Müller, and Christian Mølgaard (*The Journal of Clinical Endocrinology & Metabolism* **84**:4591–4596), Table 1 was incorrectly reproduced. Under the heading "whole body percent fat," in the line entitled "mean z-score (95% CI)," the footnote symbols are incorrect. Reading left to right the symbols *a*, *c*, *a,c*, and *a,c* should read *a*, *b,c*, *a,b*, and *a,c*. Footnote *c* should be labeled *b,c*. The printer regrets the error.

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Oestrogen-mediated suppression of tumour necrosis factor alpha-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens☆

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Abstract

In oestrogen receptor (ER)-positive breast carcinoma cells, 17β-oestradiol suppresses a dose-dependent induction of cell death by tumour necrosis factor alpha (TNF). The ability of oestrogens to promote cell survival in ER-positive breast carcinoma cells is linked to a coordinate increase in Bcl-2 expression, an effect that is blocked with the pure anti-oestrogen ICI 182,780. The role of Bcl-2 in MCF-7 cell survival was confirmed by stable overexpression of Bcl-2 which resulted in suppression of apoptosis induced by doxorubicin (DOX), paclitaxel (TAX) and TNF as compared to vector-control cells. The pure anti-oestrogen ICI 182,780 in combination with TNF, DOX or TAX potentiated apoptosis in vector-transfected cells. Interestingly, pre-treatment with ICI 182,780 markedly enhanced chemotherapeutic drug- or TNF-induced apoptosis in Bcl-2 expressing cells, an effect that was correlated with ICI 182,780 induced activation of c-Jun N-terminal kinase. Our results suggest that the effects of oestrogens/anti-oestrogens on the regulation of apoptosis may involve coordinate activation of signalling events and Bcl-2 expression. © 2001 Published by Elsevier Science Ltd.

Keywords: Apoptosis; Oestrogen; Anti-oestrogen; Bcl-2; ICI 182,780; c-Jun N-terminal kinase; MCF-7 cells; Tumour necrosis factor alpha

1. Introduction

The process of apoptosis is controlled by expression or activation of numerous apoptotic regulatory proteins including caspases, mitogen activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB), and members of the Bcl-2 family [1,2]. Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissues [3,4]. Both prostate and mammary epithelial cells undergo apoptosis upon removal of testosterone and oestrogen, respectively [4–8]. This cellular dependence upon hormones for survival and proliferation extends to neoplasms arising from these tissues, as well. The MCF-7 breast cancer cell line has been shown to form tumours in nude, ovariectomized mice only in the presence of oestrogen [7,9]. Upon removal of oestrogen or with antagonism by anti-oestrogens, these malignant cells begin to undergo

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apoptosis leading to tumour regression [7,10–12]. Additionally, we along with others, have shown that pre-treatment of MCF-7 cells with oestrogen grown in vitro suppresses apoptosis induced by tumour necrosis factor alpha (TNF) and chemotherapeutic drugs, including tamoxifen [13–16]. These studies provide evidence that oestrogens play a role in both tumourigenesis and drug resistance through the suppression of apoptosis. The addition of anti-oestrogens, such as tamoxifen or ICI 182,780, has been shown to induce apoptosis in these cells presumably through the inhibition of oestrogen receptor (ER) survival signalling [13,17–19]. Reports also demonstrate that one mechanism by which oestrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis-regulating proteins whose expression has been shown to suppress apoptosis of MCF-7 cells [13–16]. The addition of ICI 182,780 or tamoxifen has been shown to block the Bcl-2-inducing effect of 17β-oestradiol [14–17,19], while overexpression of Bcl-2 or Bcl-xL has been shown to suppress apoptosis induced by chemotherapeutic drugs, Fas and TNF in MCF-7 cells [14,20,21]. Additionally, Pratt et al. have shown that Bcl-2-overexpressing MCF-7 cells form tumours in nude mice that do not undergo apoptosis upon oestrogen withdrawal [9]. The survival effects of 17β-oestradiol are thought to be mediated predominantly through enhanced expression of Bcl-2; however, the possibility that other anti-apoptotic signalling pathways may be involved has not been excluded. Regulation of Bcl-2 was demonstrated to occur independently of oestrogen response elements (EREs) within the promoter region suggesting an indirect effect of oestrogen on Bcl-2 expression [22], while Perillo et al. demonstrate this effect occurred through EREs within the coding region of Bcl-2 [23].

Recently, 17β-oestradiol's anti-apoptotic effect has been shown to involve rapid stimulation of cytoplasmic signalling cascades such as Erk, a member of the MAPK family, and the well-established anti-apoptotic AKT protein [24–30]. Additionally, regulation of the c-Jun N-terminal kinase (JNK) and p38 components of the MAPK pathway have been demonstrated to be critical to the anti-apoptotic effects of 17β-oestradiol [26–36]. Recent evidence has suggested that the activation of these early signalling events particularly Erk, mediate the survival signalling effects of 17β-oestradiol [26,27,32]. Therefore, the ability of the ER to regulate biological effects involves both non-genomic cytoplasmic signalling such as the MAPK cascade in addition to target genomic increases in expression of growth factors, early immediate genes (c-Fos) and survival factors (Bcl-2) that function to promote cell proliferation and cell survival [37,38].

In juxtaposition to oestrogen signalling, treatment of cells with anti-oestrogens such as tamoxifen or ICI 182,780 can induce apoptosis in MCF-7 cells [10,11,17–19,39–42]. The ability of tamoxifen and ICI to promote apoptosis is partially mediated through the suppression of 17β-oestradiol-induced Bcl-2 expression and suppression of other 17β-oestradiol-dependent survival pathways. Additionally, anti-oestrogen suppression of 17β-oestradiol-mediated cell survival has also been demonstrated to occur through inhibition of early MAPK signalling, suggesting the combination of both gene expression and early MAPK signalling are critical in the regulation of cell survival by the ER [26,27]. However, reports demonstrating that tamoxifen and ICI 182,780 can function to alter other signalling pathways, such as suppression of protein kinase C (PKC), calmodulin kinase II (CamKII) or glucosyl ceramide synthase activity suggests ER-independent effects may also be involved [42–47].

In this report, we investigate the role of 17β-oestradiol and ICI 182,780 in the regulation of apoptosis by TNF and chemotherapeutic drugs in MCF-7 cells. We demonstrate the ability of 17β-oestradiol to suppress TNF-induced apoptosis, but ICI 182,780 abrogates this effect. Pre-treatment with ICI 182,780 enhanced TNF-induced apoptosis, suggesting a requirement for ER in maintaining cell viability. This enhanced sensitivity may be occurring through both the suppression of ER signalling with subsequent Bcl-2 expression, as well as through an undefined increase in a high molecular weight form of Bcl-2 suggestive of phosphorylation. Additionally, we demonstrate that ICI 182,780 can function to enhance TNF-induced cell death in both normal and Bcl-2 overexpressing cells. The ability of ICI to subvert the anti-apoptotic effects of Bcl-2 was correlated with an increased activation of JNK. Consistent with the involvement of JNK in suppression of Bcl-2, we also demonstrate that overexpression of a mutant of MEKK1 which constitutively activates the JNK pathway, sensitizes Bcl-2 expressing MCF-7 cells to TNF and chemotherapeutic drug induced cell death. The ability of ICI 182,780 to sensitize MCF-7 cells overexpressing Bcl-2 to apoptosis suggests that multiple signals generated by the ER function to regulate apoptosis including regulation of MAPK signalling in addition to expression of Bcl-2.

2. Materials and methods

2.1. Cell culture and reagents

MCF-7 cells (N variant) were maintained and grown in Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (GibcoBRL, Gaitherburg, MD) and porcine insulin 1×10^{-10} M (Sigma Chemical Co., St. Louis, MO) under my-

coplasma-free conditions as previously described [48]. For oestrogen studies, MCF-7 cells were grown 3 days in DMEM (phenol red free) with 5% dextran-coated charcoal stripped FBS containing media (CS–FBS–DMEM) as above, but without insulin as previously described [49]. 17β-Oestradiol was obtained from Sigma. ICI 182,780 (7-α-[9-(4,4,5,5,5-pentafluoropentasulfiniyl)nonyl]estra-1,3,5(10) triene-3,17-β-diol was generously provided by Dr Alan E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Ethanol (100%) was used as vehicle control in experiments as well as to dissolve both ICI 182,780 and 17β-oestradiol stock solutions. The expression vectors for the constitutively active MEKK1 (ΔMEKK; pEE-CMV-Δ362MEKK1 and pEE-CMV-empty) were generously provided by Dr Dennis Templeton (Case Western Reserve University) [50].

2.2. Stable transfection of Bcl-2

MCF-7 cells (N variant) were transfected with SFFV-Bcl-2-neo vector or with SFFV-neo (5 μg DNA per 1×10^6 cells) using lipofectamine (1 μg DNA/3 μl of lipofectamine, GibcoBRL) in 0% serum-containing OptiMEM (GibcoBRL) without supplements in T-75 flasks. After 6 h of transfection, the lipofectamine/DNA containing media was removed and replaced with 10% FBS-fortified DMEM (10%-DMEM) as described above. Cells were allowed to recover for 48 h after which the media was removed and replaced with 10%-DMEM containing 400 μg/ml G418 (Sigma Chemical Co.). Cells were grown for 15 days with media being replaced with fresh 10%-DMEM (400 μg/ml G418). Cells were split from T-75 flasks into multiple 100 cm^2 dishes at differing dilutions and allowed to adhere for 24 h in 10%-DMEM without G418. Following this, media was removed and replaced with fresh 10%-DMEM with 400 μg G418/ml every 3 days until visible colonies appeared. Individual colonies were isolated using a sterile cloning ring coated with petroleum jelly and removed with PBS–EDTA (100 μl). Individually removed colonies were transferred to 24-well plates and allowed to grow as separate clones. Clones were grown and maintained in 10%-DMEM (400 μg/ml/G418) until 2×10^6 cell could be used for Western blot analysis for Bcl-2 expression.

2.3. Viability assay

Viability/cell death is determined using trypan blue exclusion as previously described [48]. MCF-7 cells were plated at 5.0×10^4 cells/ml in 10 cm^2 wells in 5% CS–FBS–DMEM. The cells were allowed to adhere for 18 h before treatment with or without 17β-oestradiol (Sigma Chemical Co.) for 24 h followed by recombinant human TNF-α (10 ng/ml; R&D systems, Minneapolis, MN). Cells were then counted at 48 h post-TNF treatment for viability assay. For Bcl-2 viability studies, cells were plated in 10%-DMEM for 18 h, and the following day media was changed to 0%-DMEM followed by treatment with or without ICI 182,780 for 60 min, followed by treatment with either TNF, paclitaxel (TAX; Biomol, Plymouth Meeting, PA), or doxorubicin (DOX; ICN, Aurora, OH). Cells were harvested 24, 48 and 72 h later, and viability was measured by trypan blue exclusion. The results are represented as the number of viable cells/ml. Apoptosis is expressed as the percentage of trypan blue-stained cells in treated samples compared to control viability (100%).

2.4. DNA fragmentation analysis

Following treatment, cells were harvested for DNA as described previously [48]. Briefly, $1–2 \times 10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris–HCl, 10 mM EDTA, 0.5% SDS (w/v) pH 7.4] to which RNAse A (100 μg/ml) was added. After incubation for 2 h at 37 °C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56 °C for 1 h. Sodium chloride (NaCl) was then added (final concentration, 1 M) and lysates were incubated overnight at 4 °C. Lysates were centrifuged at $15{,}000 \times g$ for 30 min, and nucleic acids in the supernatant were precipitated in two volumes of ethanol with 50 mM Na acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by staining with ethidium bromide.

2.5. Western blot analysis

MCF-7 cells were grown for 2 days as described above, and then 5×10^6 cells were harvested in sonicating buffer (62.5 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 1 mM Na Orthovanadate) and sonicated for 30 s. Following centrifugation at $1000 \times g$ for 20 min, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS–Tween (0.05%)-5% low fat dry milk solution at 4 °C overnight. The membrane was subsequently incubated with rabbit Bcl-2 antisera (1:4000) [48,51] (generously provided by John Reed), with monoclonal antibodies to Bcl-2 (1:1000) [48] (Pharmingen, San Diego, CA), or phospho-specific monoclonal antibodies to P-Erk1/2 (1:1000), P-JNK1/2 (1:1000), P-p38α (1:1000; New England Biolabs, Beverly, MA). Follow-

ing incubation for 2 h at room temperature, blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat anti-mouse antibodies conjugated to horseradish peroxidase (1:5000 dilution; Oxford, Oxford, MI) for 60 min at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm (Amersham), according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad, Hercules, CA).

3. Results

Several reports have demonstrated that oestrogens promote cell survival in oestrogen-responsive cells [13–16]. We examined the ability of 17β-oestradiol to promote cell survival in MCF-7 cells treated with TNF (Fig. 1(a)). TNF is a potent inducer of apoptosis in MCF-7 cells resulting in a decrease in viability to 59 ± 5.9, 41.9 ± 4.6 and $39.4 \pm 5.4\%$ with 0.1, 1.0 and 10 ng/ml, respectively, at 24 h after treatment. Consistent with previous results [49], pre-treatment of MCF-7 cells with 1 nM 17β-oestradiol for 24 h circumvents a dose-dependent induction of cell death by TNF (0.1–10 ng/ml) to 72.6 ± 3.1, 61.2 ± 4.3 and $60.0 \pm 2.4\%$ viability. Interestingly, 1 nM 17β-oestradiol pre-treatment for only 1 h was sufficient to partially suppress TNF-induced cell death (data not shown). No difference in viability was observed between vehicle alone and oestradiol alone. The ability of 17β-oestradiol to suppress TNF-induced apoptosis was blocked by the pre-treatment with the pure anti-oestrogen ICI 182,780 (1 μM), restoring TNF-induced cell death (Fig. 2(a)). The ability of 17β-oestradiol to suppress and ICI 182,780 to restore TNF-induced apoptosis was demonstrated using DNA fragmentation analysis (Fig. 2(b)). These studies also demonstrate that ICI 182,780 alone is capable of slightly increasing DNA-fragmentation as compared to vehicle treated control cells. The ability of 17β-oestradiol to suppress cell death has been correlated with

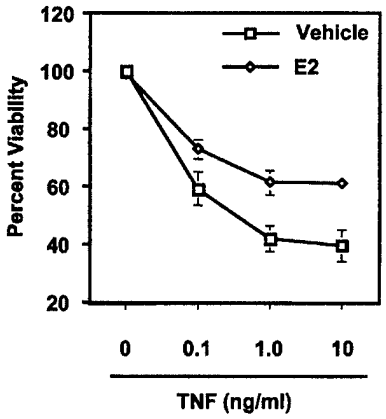

Fig. 1. Oestrogen suppression of TNF induced cell death. MCF-7 cells were plated in 5% CS-containing media for 18 h followed by treatment with or without 17β-oestradiol (1 nM) for 24 h. Cells were then treated with TNF (0.1–10 ng/ml) for 24 h and harvested for viability. Data are represented as the mean ± SEM > of six experiments.

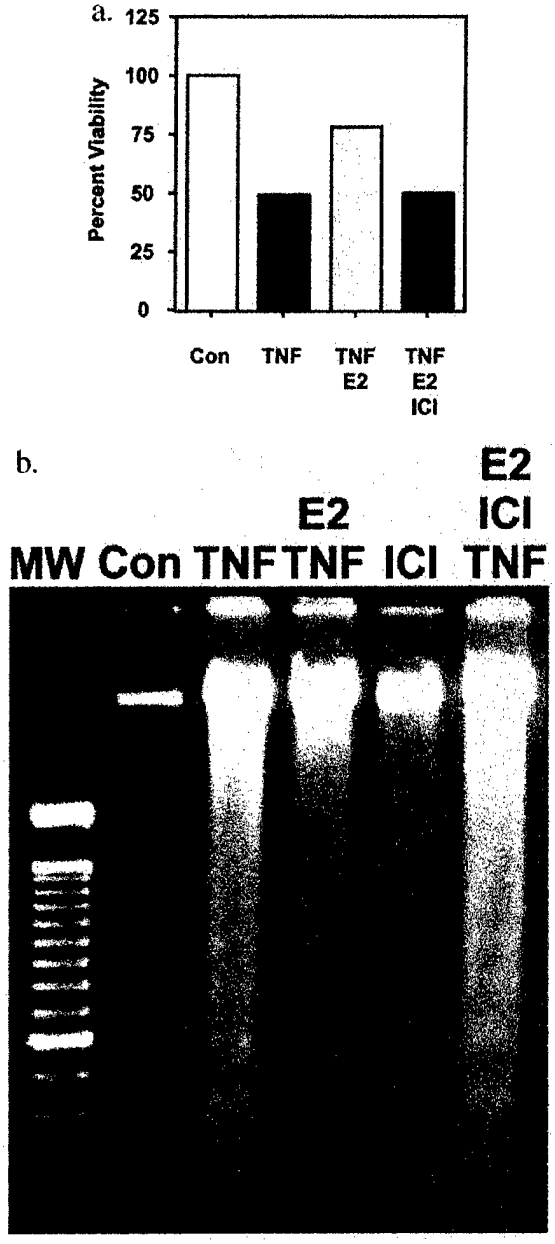

Fig. 2. Suppression of oestrogen-mediated cell survival by ICI 182,780. MCF-7 cells were plated in 5% CS media for 18 h, pre-treated with vehicle (Con) or ICI 182,780 (1 μM; ICI 182,780) for 30 min followed by vehicle or 17β-oestradiol (1 nM; E2) for 24 h. Cells were then treated with TNF (10 ng/ml) for 24 h and harvested for viability assay (a) or DNA fragmentation analysis (b). Data displayed are representative of three experiments.

increased expression of Bcl-2. Consistent with these reports, 1 nM 17β-oestradiol was shown to increase expression of Bcl-2, as compared to vehicle treated controls cells (Fig. 3). Additionally, ICI 182,780 suppressed both basal and 17β-oestradiol-stimulated induction of Bcl-2 expression. Interestingly, an increased level of the 32 kDa molecular weight Bcl-2 was observed prominently with ICI 182,780 treatment. Previous studies have demonstrated this to be a phosphorylated form of Bcl-2 [52–55], suggesting an increased phosphorylation of Bcl-2 upon ICI 182,780 treatment. Bcl-2 expression has been associated with significant resistance to apoptosis by numerous agents. However, contradictory reports [20,56] addressing the role of Bcl-2 in resistance to apoptosis exist regarding MCF-7 cells, and we wished to determine if Bcl-2 suppressed apoptosis in the MCF-7 cell variant used in our studies.

MCF-7 (N variant) cells were transfected with a pSFFV-Bcl-2 construct or empty vector (Fig. 4). Cells were selected in DMEM-10% media containing G418 and clones were examined for Bcl-2 expression. All vector-transfected clones examined expressed very low to undetectable levels of Bcl-2. In contrast, all SFFV-Bcl-2 transfected clones (B1-6 represented) displayed significant levels of Bcl-2 levels. Vector and Bcl-2 containing clones were compared to another variant of MCF-7 (M variant) cells. The MCF-7M cells were previously shown to endogenously express high levels of Bcl-2 protein compared to the MCF-7N cells used here [48]. Arbitrarily, MCF-7N-Bcl-2 (B3; MCF-7/Bcl-2) and MCF-7N-Vec (MCF-7/Vec) were selected to examine the effects of Bcl-2 expression on TNF-, TAX- and DOX-induced apoptosis. Both clones were examined for sensitivity to either TNF-, DOX- or TAX-mediated cell death at 24, 48 and 72 h (Fig. 5(a), (b) and (c)). These results demonstrate that TNF treatment resulted in a potent loss of viability in MCF-7N-Vec cells with a 63 ± 5.5, 37 ± 11.7 and $23 \pm 10.7\%$ viability at 24, 48 and 72 h post-treatment, respectively. However, MCF-7/Bcl-2 cells were resistant to TNF-induced cell death with only a slight loss of viability to 90 ± 5.7, 87 ± 3.8 and $86 \pm 7.6\%$ at 24, 48 and 72 h post-treatment, respectively. Similarly, both DOX and TAX treatment resulted in significant cell death in MCF-7/Vec cells. TAX produced a loss of viability to 69 ± 1.2, 67 ± 1.7 and $60 \pm 0.68\%$ at 24, 48 and 72 h post-treatment, respectively, while DOX-induced a loss of viability to 68 ± 4.2, 49 ± 3.1, 35 ± 8.5 at 24, 48 and 72 h, respectively. In contrast, MCF-7/Bcl-2 cells displayed resistance to cell death with a viability of 97 ± 3.1, 99 ± 0.5 and $95 \pm 2.5\%$ with TAX at 24, 48 and 72 h post-treatment, respectively, and percent viability of 99 ± 1.1, 94 ± 6 and $75 \pm 4.6\%$ with DOX at 24, 48 and 72 h post-treatment, respectively. Increased expression of Bcl-2 protein levels by oestrogens has been a proposed mechanism by which this steroid hormone promotes cell survival. Interestingly, the ability of ICI 182,780 to suppress Bcl-2 expression also revealed an increase in a higher (32 kDa) molecular weight form of Bcl-2 suggestive of phosphorylation, an event associated with decreased anti-apoptotic activity of this protein. Using MCF-7/Bcl-2 and MCF-7/Vec cells, we wished to examine the possibility that ICI 182,780 may function to partially suppress the survival effect of Bcl-2 in the MCF-7/Bcl-2 cells or enhance apoptosis in the MCF-7/Vec cells. Specifically, MCF-7/Vec cells were pre-treated with ICI 182,780 (1 μM) for 1 h, followed by treatment with TNF (10 ng/ml), TAX (0.04 μg/ml) or DOX (0.1 μg/ml). Subsequent viability outcomes demonstrated that the combined TNF and ICI 182,780 (TNF/ICI) treatments resulted in a decrease in viability to 21 ± 4.6, 6.6 ± 3.4 and $8 \pm 2.6\%$ at 24, 48 and 72 h post-treatment in MCF-7/Vec cells, respectively, while the TAX/ICI-treated MCF-7/Vec cells resulted in a loss of viability to 53 ± 1.7, 47 ± 4 and $37 \pm 6\%$ at 24, 48 and 72 h post-treatment, respectively. The DOX/ICI-treated MCF-7/Vec cells resulted in a loss of viability to 43 ± 4.7, 18 ± 3.8 and $35 \pm 20\%$ at 24, 48 and 72 h post-treatment, respectively. Similar to

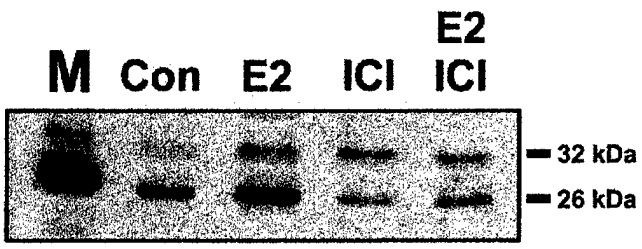

Fig. 3. Regulation of Bcl-2 expression and phosphorylation by oestrogen signalling. MCF-7 cells were plated in 5% CS media for 18 h, pre-treated with or without ICI 182,780 (1 μM; ICI) for 30 min followed by 17β-oestradiol (1 nM; E2) for 24 h. Cells were then harvested for Western blot analysis of Bcl-2 expression using Bcl-2 antisera. High Bcl-2-expressing MCF-7M cells were used as a standard (M).

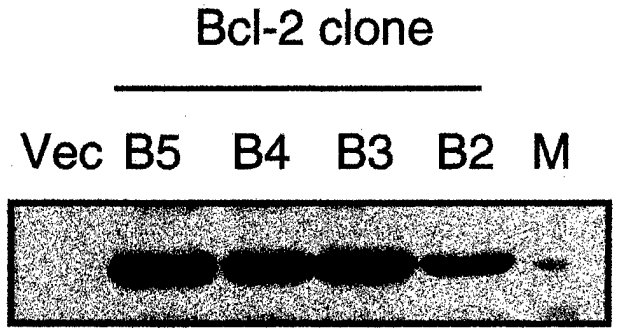

Fig. 4. Generation of stable Bcl-2 overexpression in MCF-7 cells. MCF-7 cells were transfected with either pSFFV-Bcl-2 or empty vector. Selected clones of MCF-7N-Vec and MCF-7N-Bcl-2 were examined for Bcl-2 expression by Western blot analysis. High Bcl-2-expressing MCF-7M cells were used as a standard (M).

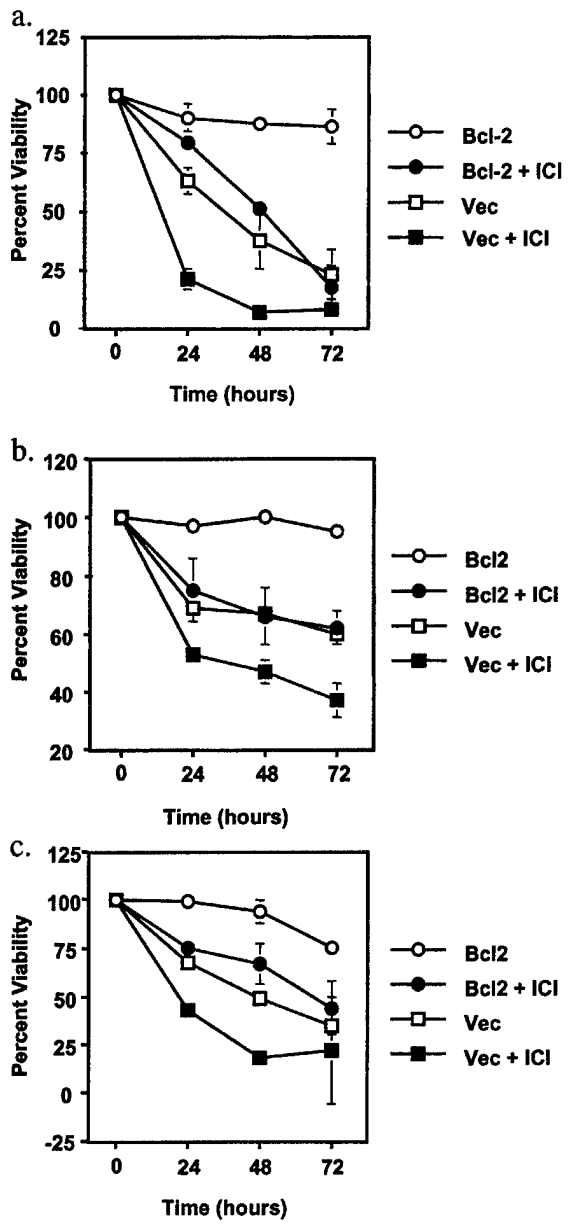

Fig. 5. Bcl-2-mediated suppression of TNF-, TAX- and DOX-induced cell death and reversal by anti-oestrogens. MCF-7N/Vec (clone 1; squares) and MCF-7N/Bcl-2 (clone 3) cells (circles) were treated with or without ICI 182,780 (10 μM; filled) for 1 h followed by treatment with either TNF (10 ng/ml) (a), TAX (0.04 μg/ml) (b) or DOX (0.1 μg/ml) (c) for 24, 48 or 72 h. Cells were then harvested for viability assay. Data are representative of three independent experiments ± SEM.

the MCF-7/Vec cells, the MCF-7/Bcl-2 cells pre-treated with ICI 182,780 also showed an increased loss of viability when compared to control MCF-7 cells. Specifically, viability outcomes from the TNF/ICI treatments resulted in a decrease in viability to 79 ± 1.7, 51 ± 2.1 and 17 ± 9.3% at 24, 48 and 72 h post-treatment in MCF-7/Bcl-2 cells, respectively, while the TAX/ICI-treated MCF-7/Bcl-2 cells resulted in a loss of viability to 75 ± 10.6, 66 ± 9.8 and 62 ± 5.7% at 24, 48 and 72 h post-treatment, respectively. The DOX/ICI-treated MCF-7/Bcl-2 cells resulted in a loss of viability to 75 ± 0.58, 67 ± 10.4 and 44 ± 14% at 24, 48 and 72 h post-treatment, respectively.

The ability of ICI 182,780 treatment to subvert the anti-apoptotic effect of Bcl-2 overexpression suggested that suppression of an ER-dependent survival signal or activation of an apoptotic signal by ICI 182,780 was capable of subverting cell survival mediated by Bcl-2. Several studies have shown that activation of JNK is capable of phosphorylating and inactivating Bcl-2. The ability of ICI to activate a Bcl-2 phosphorylating cascade such as JNK may be in part responsible for the apoptotic enhancement seen with ICI in Bcl-2 over expressing cells. Previous reports have indicated that oestrogen treatment mediates cell survival through rapid activation of Erk signalling and recently, a coordinate suppression of JNK activation [26,27,33–36]. Additionally, tamoxifen has been shown to both suppress oestrogen-ER activation of Erk as well as to stimulate JNK activation [33–36]. The regulation of early MAPK singling by oestrogen–anti-estrogens may be critical in cell survival independently or in conjunction with oestrogen stimulation of Bcl-2 expression. We, therefore, examined the ability of ICI 182,780 treatment to activate the Erk, JNK and p38 members of the MAPK family (Fig. 6). Our results showed that in a time-dependent manner (0–60 min), ICI 182,780 (1 μM) treatment resulted in a rapid increase in phosphorylation of JNK and p38. In contrast, ICI 182,780 resulted in a rapid partial suppression of basal Erk phosphorylation.

The observation that ICI 182,780 treatment results in increased activation of JNK suggests a potential role for this pathway in mediating the survival subverting effect of ICI in MCF-7/Bcl-2 (clone 3) cells. Using a constitutive active mutant of MEKK1 (ΔMEKK) which has been previously shown to potently stimulate the JNK cascade [50], we investigated the ability of

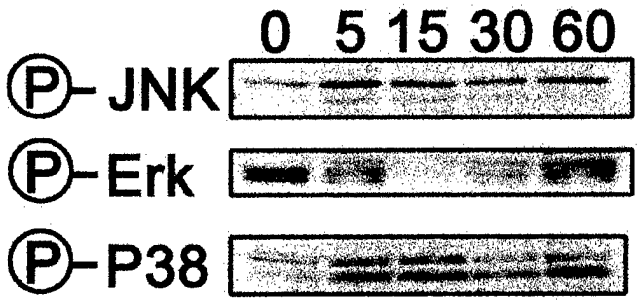

Fig. 6. Activation of JNK by the anti-oestrogen ICI 182,780. MCF-7 cells were treated with ICI 182,780 (1 μM), harvested at the times indicated and analysed for the activation of Erk, JNK and p38 MAPKs. Phosphorylated forms of Erk 1,2 (P-Erk), JNK 1,2 (P-JNK) or p38 (P-P38) were detected using immunoblot analysis with phospho-specific antibodies as indicated in Section 2. The figure is a representative blot of three experiments.

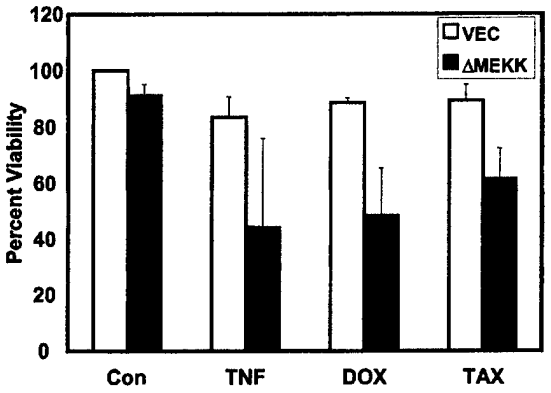

Fig. 7. MEKK activation potentiates TNF-, TAX and DOX-induced cell death in MCF-7/Bcl-2 cells. MCF-7/Bcl-2 (clone 3) cells were transfected with 3 μg of empty pEE-CMV vector (VEC) or with constitutive active MEKK, pEE-CMV-ΔMEKK1 (ΔMEKK), using the lipofectamine method followed by treatment with either 10 ng/ml TNF, 0.04 μg/ml TAX, or 0.1 μg/ml DOX and harvested 24 h later for viability assay. Data are represented as percent viability compared to untreated (CON) VEC transfected cells (100%) from two independent experiments ± SEM.

MEKK–JNK activation to subvert Bcl-2 cell survival. MCF-7/Bcl-2 (clone 3) cells were transfected with either empty vector or a ΔMEKK construct and treated with TNF, TAX or DOX (Fig. 7). Consistent with the above results, MCF-7/Bcl-2 (clone 3) cells exhibited weak responses to TNF (83.5 ± 7.5), TAX (88.5 ± 1.6) or DOX (89.1 ± 5.6) treatment. In contrast, expression of the ΔMEKK in MCF-7/Bcl-2 (clone 3) cells markedly enhanced the ability of TNF (44.2 ± 31.5), TAX (48.5 ± 16.6) or DOX (61.5 ± 10.7) to induce cell death. This suggests that molecular activation of the MEKK–JNK pathway, similar to ICI 182,780 treatment, subverts the survival effect of Bcl-2.

4. Discussion

Oestrogen represents a potent survival factor in a number of biological systems [3,32]. In the MCF-7 breast carcinoma cell line, numerous reports have implicated oestrogen and ER-signalling in the suppression of apoptosis [7,10–16]. However, treatment of MCF-7 cells with anti-oestrogens, such as tamoxifen and ICI 182,780, is associated with the induction of apoptosis in these cells [14–17,19,57]. Similarly, removal of the oestrogen source in nude, ovariectomized mice results in apoptosis of MCF-7 tumour implants [7,19]. This phenomenon suggests that oestrogen may play a permissive role in cell proliferation and survival, similar to the role of cytokines, such as IL-3 and GM-CSF, in haematopoietic cell survival [58]. Therefore, in the presence of oestrogen, breast carcinoma cells are allowed to proliferate, but upon the removal of oestrogen or upon ER-signalling inhibition, these cells die by the loss of a necessary survival signal. The ability of oestrogen to induce cell survival is, in part, dependent upon Bcl-2 expression. In agreement with previous results, we demonstrate in this report that an increase in Bcl-2 expression is seen with oestrogen treatment, which is suppressed by ICI 182,780 treatment. ICI 182,780 not only suppresses 17β-oestradiol-induced Bcl-2 expression, but ICI 182,780 also appears to increase the presence of a 32 kDa higher molecular weight form of Bcl-2 previously described as a phosphorylated form of Bcl-2. Phosphorylation of Bcl-2 has been associated with decreased anti-apoptotic effects by the Bcl-2 molecules themselves [52–55]. The ability of ICI to suppress 17β-oestradiol-induced Bcl-2 expression is expected, however, the observed increase in a higher molecular weight Bcl-2 form suggestive of phosphorylation is an interesting and novel observation. These effects of ICI 182,780 may be in part due to additional ER-mediated, survival-signalling pathways. Recently, a report by Dong et al. demonstrated that oestrogen-mediated activation of Bcl-2 expression did not occur through direct regulation of the Bcl-2 promoter by ER, but rather through an oestrogen-dependent induction of cAMP, which then stimulates Bcl-2 expression through a CREB/ATF-I-dependent activation of cAMP-response-elements (CREs) [22]. However, a report by Perillo et al. demonstrated an ER-dependent regulation of Bcl-2 through ERE sites within the coding region [23]. The potential exists that Bcl-2 may only represent one pathway by which oestrogen and the ER function to promote cell survival. Therefore, the effects of oestrogens on survival signalling may occur through activation of other cellular signalling cascades.

Several reports have demonstrated that 17β-oestradiol can rapidly stimulate Erk activation in MCF-7 and other cell lines and is involved in mediating anti-apoptotic effects of 17β-oestradiol [26–30,32]. In contrast to Erk signalling to cell survival, the JNK and p38 members of the MAPK cascade have been demonstrated to induce or enhance apoptosis in many systems. Also of note is a recent report by Zhang et al. that demonstrates an increased activation of p38 MAPKs with 17β-oestradiol treatment [36]. Collectively, these results demonstrate the ability of Erk, JNK and p38 MAPKs to be regulated by ER signalling. Evidence now suggests that 17β-oestradiol signalling to cell survival involves both a rapid up-regulation of Erk activation coordinate to suppression of JNK activity [26,27]. Therefore, given that Erk may be an important survival signal, the ability of ICI 182,780 or tamoxifen to suppress 17β-oestradiol-induced Erk activities may inhibit ER-survival signalling. Additionally, treatment with ICI 182,780 or tamoxifen may block 17β-oestradiol's effects on JNK thus leading to both a decrease in Erk activation while increasing JNK activation.

The involvement of these rapid signalling events in the regulation of cell survival are consistent with our observation that a 1 h pre-treatment with 17β-oestradiol results in partial suppression of TNF-induced apoptosis. The ability of ICI 182,780 treatment to suppress the anti-apoptotic effects of Bcl-2 overexpression suggests both gene expression and rapid signalling are critical to the survival effects of 17β-oestradiol. Consistent with the involvement of the MAPK pathways in regulation of survival by the ER, we observed that ICI 182,780 treatment rapidly enhances JNK activation in MCF-7 cells. Activation of the JNK pathway has been demonstrated to induce Bcl-2 phosphorylation, an event that suppresses the anti-apoptotic effects of Bcl-2 [53,55]. Consistent with these findings we observed ICI treatment induced an increased molecular weight band of Bcl-2 suggestive of phosphorylation. Therefore, direct promotion of Bcl-2 phosphorylation by ICI 182,780 or tamoxifen [59] may be through a JNK-dependent pathway or through an indirect suppression of the 17β-oestradiol/ER-dependent pathway that functions to maintain Bcl-2 in an unphosphorylated state. This concept is supported by the ability of ICI 182,780 to override the Bcl-2-mediated survival signal. It has however been previously demonstrated that tamoxifen can suppress kinases, such as PKC and CamKII, as well as glucosyl ceramide synthase, and that this may be a mechanism by which tamoxifen or ICI 182,780 can influence other ER-independent effects in cells [42–47]. Activation or suppression of these other pathways by ICI 182,780 treatment may be similar to the ability of TAX to increase Bcl-2 phosphorylation, and thereby shift the survival balance towards cell death [52,54].

In conclusion, our results demonstrate a critical role for ER signalling in MCF-7 cell survival and suppression of chemotherapeutic drug- and TNF-induced apoptosis. Additionally, our observations demonstrate that ICI 182,780 functions to suppress both oestrogen- and Bcl-2-mediated cell survival in ER-positive cells, suggesting the existence of both gene expression (Bcl-2) and cytoplasmic signalling (MAPK) mechanisms of ER-mediated cell survival in MCF-7 cells.

Acknowledgements

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From malformations to molecular mechanisms in the male: three decades of research on endocrine disrupters

Review article

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For three decades, we have known that estrogens alter the development of the mammalian reproductive system in predictable ways. In mice exposed prenatally to diethylstilbestrol (DES) or other estrogens, the male offspring exhibit structural malformations including cryptorchidism, epididymal cysts and retained Mullerian ducts. The estrogen-associated alterations in the genital tract phenotype can be usefully considered as a model called Developmental Estrogenization Syndrome. While estrogen treatment during critical periods of morphogenesis of the male reproductive system has been associated with these changes, the mechanisms at the molecular level are still being discovered. Parallel findings on the hormones involved in Mullerian duct regression and testicular descent have helped guide research on the mechanisms of developmental estrogenization of the male. Cellular localization of molecular signals associated with key steps in genital tract development, use of mice with gene disruption, and knowledge of the mechanisms underlying persistent changes in gene expression are beginning to provide a blue print for both the physiological role and pathological effects of estrogens in reproductive tract development. Since many of the same biological principles underlie genital tract morphogenesis in mammals, one may expect some of the same changes in males of other species exposed to estrogen during the appropriate developmental periods.

Key words: Malformations; male, estrogens; reproductive system; molecular mechanisms.

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Science usually works to put what we know or observe into some semblance of order. Seldom does science go beyond what "we know to be true" at any given time; when it does, we usually look back and consider such events as scientific breakthroughs.

Thirty years ago a paper by Arthur Herbst and his colleagues (1) changed the way we think about the effects of drugs and other chemicals on the developing human fetus. Herbst linked the occurrence of rare cervical and vaginal cancers in a small group of young women who were treated by him at the Massachusetts General Hospital to the ingestion by their mothers of a synthetic estrogen, diethylstilbestrol, DES, during their own pregnancies. This was the first example of transplacental carcinogenesis in

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humans. In other words, a chemical is transferred from the maternal circulation, across the placenta, into the fetal circulation and causes a change in the developing vaginal cell, resulting in cancer after the developing fetus is born. In the case of DES and vaginal cancer, this was usually 15 to 30 years after the exposure in utero.

The three decades of research stimulated by that clinical paper have not only provided mechanisms to explain the initial clinical observation, but also opened a wholly new avenue of understanding about hormones, our chemical environment and embryonic development. In trying to understand the DES observation we have learned about steroid hormone structure and function, the role of metabolism in hormonal carcinogenesis, the mechanism of differentiation of the mammalian reproductive tract and the structural diversity of estrogenic chemicals.

Moreover, it was these new areas of discovery associated with the effects of DES on the mammalian fetus that have led to what are now called endocrine disrupting chemicals or environmental hormones. The impetus for study of estrogens in the environment and the subsequent convergence of observations in clinical, laboratory and field studies can, to a large extent, be attributed to DES.

Diethylstilbestrol is a chemical with potent estrogenic properties synthesized by Dodds and colleagues in 1938 (2). Because DES is potent, orally active and relatively inexpensive to manufacture, it has found numerous uses in the 60 years since its synthesis. It has been used to caponize chickens, increase weight gain in cattle, treat prostate cancer in men, suppress lactation and prevent miscarriage in women (3).

From 1948 to 1976, DES was prescribed to pregnant women in many countries including the United States, England, Holland, Denmark and Finland. It is estimated that 4 to 8 million pregnancies were treated with DES in the United States alone.

Scientific knowledge 30 years ago led to three assumptions: 1) chemicals that could reach the fetus on rare occasion would, if they had any deleterious effect, cause structural abnormalities recognized at birth; 2) estrogens were "natural" materials in the body, so synthetic hormones should also behave naturally; 3) the fetal compartment was known to contain estrogens from the mother, so more estrogens should not be deleterious.

While maternal ingestion of DES was not associated with noticeable, external, anomalies in the offspring at birth, cancers were detected in female offspring more than a decade later. The implications of these findings led the scientific and public media to call DES exposure in utero, the equivalent of a "biological time bomb". The fuse is lit by DES in utero, but the "bomb", in the form of vaginal cancer, goes off 20 years later. The assumptions of that time were in need of change.

DEVELOPMENTAL ESTROGENIZATION SYNDROME

In 1975, we described a suite of structural defects in male CD-1 mice exposed prenatally to DES (4). These abnormalities in the reproductive system included retained testes, epididymal cysts, distended seminal vesicles and retained mullerian ducts (Fig. 1). The animals were also shown to have abnormal sperm, reduced fertility and increased testicular cancer (5, 6). The severity of these changes was dose dependent as was the appearance of all the lesions in the suite. We subsequently showed that the epididymal cysts were of Mullerian duct origin (7); it was apparent that the enlarged prostatic utricle was also the Mullerian contribution to the prostate gland.

Subsequent studies from our lab and others helped define a phenotype typical of male mice exposed in utero to DES and other estrogens. The structural or functional changes associated with the phenotype usually included undescended testes, cysts of the epididymis, prostatic lesions, and abnormal spermatogenesis (even in a scrotal testis). In a smaller number of cases, the occurrence of testicular cancers was noted. The results of Sharpe & vom Saal (8) and others who have studied the effects of steroidal and environmental estrogens on the genital tract of the fetal male rodent have provided further confirmation that estrogen can induce long-term functional changes. More recently, the work of Korach's group on the estrogen receptor alpha null or ERα knock out (ERKO) mouse (9) has added strong support to the concept that male

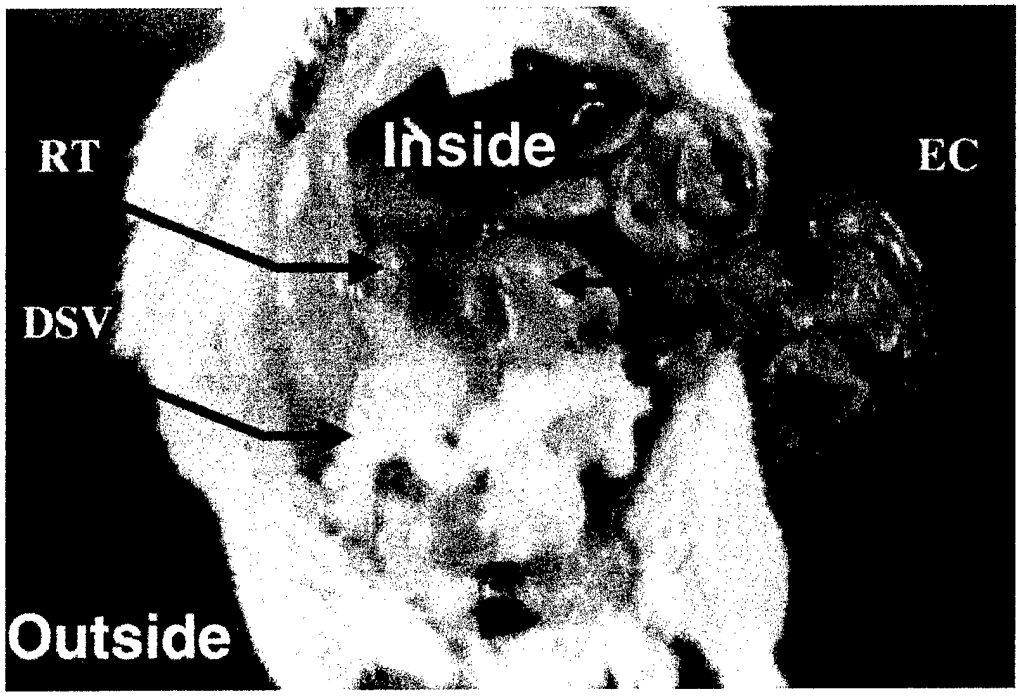

Fig. 1. Structural changes in the genital tract of a male mouse treated prenatally with DES. This phenotype constitutes the Developmental Estrogenization Syndrome. RT=retained testis, EC=epididymal cysts, and DSV=distended seminal vesicles.

genital tract development may have an estrogen component; dysgenesis of the testis was found in the ERKO males.

To account for the common features as well as provide a construct for both mechanistic studies and comparison with other species, including humans, we have chosen the term Developmental Estrogenization Syndrome to refer to the phenotype described.

The similarity in the morphogenesis of the reproductive system in mammals as diverse as the mouse and human provides comparative insights into the spectrum of effects one might associate with in utero estrogen exposure in either. As seen in Fig. 2, mice and human fetuses progress from an "indifferent" stage of internal genitalia in which both the presumptive male (Wolffian duct) and female (Müllerian duct) reproductive organs coexist regardless of the genetic sex of the fetus to the definitive structure of the appropriate gender. This process is under the control of hormones from the fetal testes following differentiation of the fetal gonad. The configuration will be female unless the fetal testis intervenes by secreting Müllerian Inhibiting Substance (MIS) to induce regression of the Müllerian duct and testosterone to maintain the Wolffian duct (for review see (10)).

In mice, DES exposure in utero results in the retention of both male and female genital ducts, thus forming a male pseudohermaphrodite or a genetic male with functioning testes and male genital tract as well as a female genital tract. Failure of testicular descent is also commonly observed.

Studies in organ culture confirm the retention of female genital anlage in the DES-exposed tissues and extend the in vivo observations to demonstrate that the DES effect is not on the synthesis or secretion of MIS from the fetal testes, but in the Müllerian duct resistance to the apoptotic signal of MIS (11).

In support of the work which suggested that the fetal Müllerian duct was a primary target for DES in utero were studies done to determine the distribution of radiolabeled DES in pregnant mice (12). We showed the transfer of DES from the maternal circulation to that of the fetus. It was further demonstrated that once in the fetal compartment, DES and its metabolites

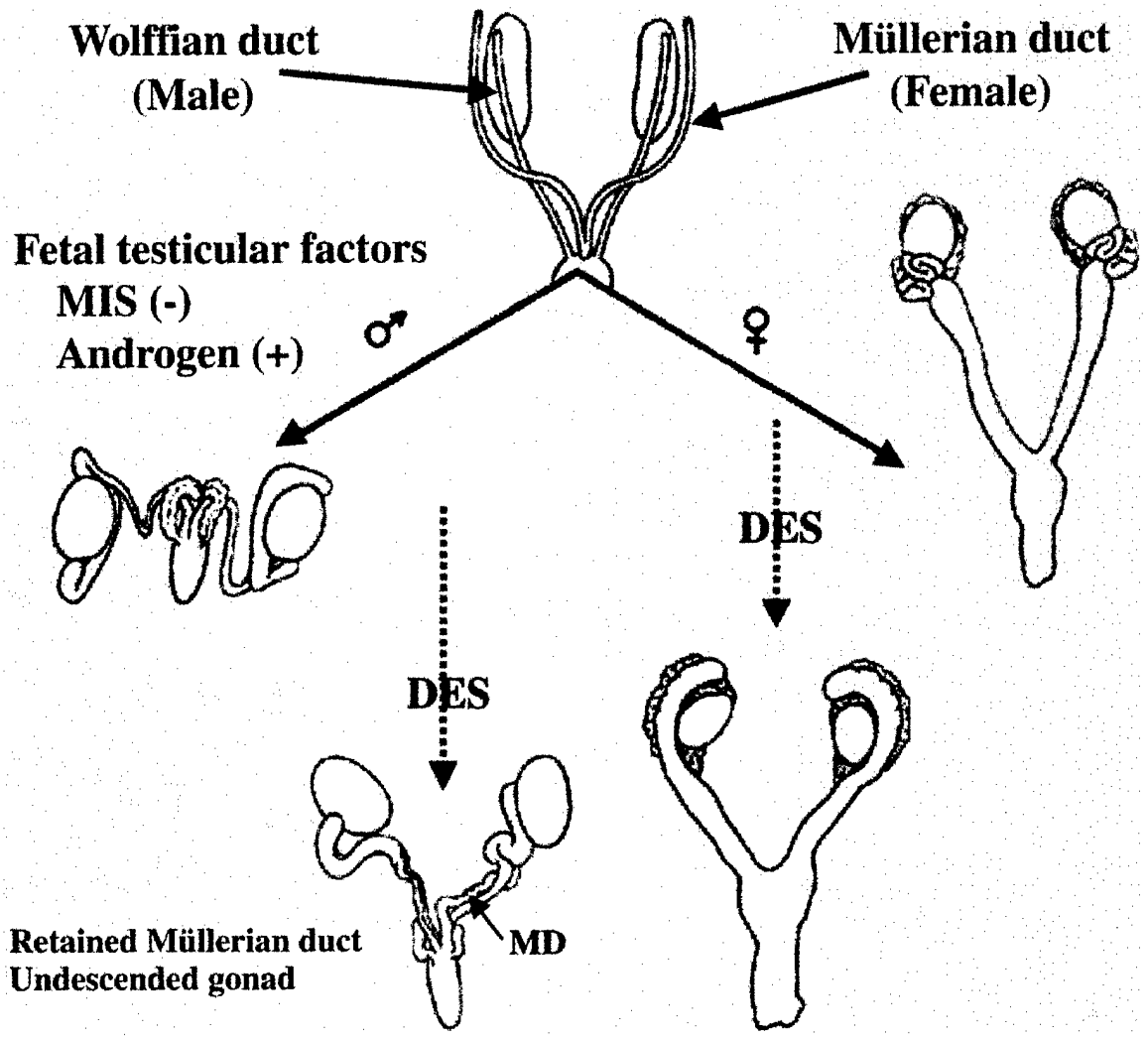

Fig. 2. Mechanisms associated with sexual differentiation of the mammalian reproductive system.

accumulated 3 fold in the fetal genital tract, but not other fetal organs (12). We subsequently showed that the fetal reproductive tract in organ culture formed metabolites known to proceed by activating metabolism (13). Finally, we were able to show by competitive binding assays and sucrose density gradient centrifugation that DES was not a high affinity ligand for alpha feto protein (AFP), while estradiol was; thus, since DES was not significantly retained extracellularly, one could conclude that DES had access to the fetal compartment while estradiol had not (14). The role of extracellular binding proteins in determining the relative availability of estrogenic chemicals in relation to their developmental toxicity continues to be an important topic (8, 15, 16).

Features in the human male

In 1975, the same year as our report on the effects of prenatal exposure to DES on the male offspring in mice (4), studies were published in which genital defects were also observed in men whose mothers had taken DES (17, 18). The group at University of Chicago reported DES-exposed men had a higher incidence of undescended (cryptorchid) testes and epididymal cysts than comparable unexposed men. Gill and his colleagues (19, 20) went on to confirm and extend these studies and showed, in addition, a

higher incidence of hypoplastic testes and abnormal sperm. In one of their papers the finding of testicular cancer in one DES-exposed man was reported and the possibility of cancer of the testis as a result of prenatal exposure to DES is raised by Gill et al. (20). A few other case reports of testicular cancer (seminoma) and epididymal cysts in prenatally DES-exposed men were reported (for example (21)).

Comparison of the mouse and human data demonstrated the importance of understanding the timing of biological events involved in the development of the reproductive tract of each. For example, when comparing the total dose of DES administered during pregnancy in the mouse compared to the human to produce retained testes, the Relative Potency Index was over 80. However, when the dose comparison was made during the biologically relevant period for testicular descent in both species (days 14–16 of gestation in the mouse and weeks 7 to 27 in the human) the RPI was now between 1 and 2 (22).

Thus, the male offspring of DES-exposed pregnancies of both mice and humans shared some defects in common. These were undescended testes, epididymal cysts and sperm abnormalities. A recent study by Wilcox's group confirmed the occurrence of structural abnormalities in DES-exposed men, but reported that there was not a significant difference in fertility between the cases and controls (23). While retention of the female genital anlage, the Müllerian duct, was a prominent feature in DES-exposed male mice, no report from similarly exposed men has addressed this issue. One might expect some element of Müllerian duct retention in the human male, since the hormone responsible for regression of the female duct is also thought to play a role in testicular descent, a defect common to both species.

The defects in morphogenesis of the male reproductive tract of mice with similarities to humans exposed to DES in utero anticipated and supported an environmental component for the elegant expression of the clinical syndrome described by Skakkebaek as Testicular Dysgenesis Syndrome (see 24).

Moreover, as with most experimental studies, the strength of a phenotypically coherent model, such as a Developmental Estrogenization Syndrome, lies in its predictability. For example, the model suggests that prenatal estrogen levels would be associated with later genital anomalies. One way to explore that issue is to ask what the estrogen status was during the fetal development of men with testicular cancer or cryptorchidism. This approach has been used successfully in evaluating the prenatal contribution of steroid hormones to subsequent risk for breast cancer. We examined four papers in which the maternal pregnancy conditions were recorded for men with either testicular cancer or crytorchidism and noted that the clinical conditions reported, obesity, hyperemesis, first pregnancy or hypertension, were each associated with elevated estrogen levels in their mothers (25).

GENE IMPRINTING WITH ESTROGEN

Another way to use a model such as the Developmental Estrogenization Syndrome is as a guide for mechanistic studies. To that end, we asked if genital tract development was altered by estrogen at the molecular level.

Natural reporter genes for hormone action during development

To investigate at this level, our group had to first make two biological reagents that we could use to study differentiation and function of Wolffian (male) duct derivatives as well as that of Müllerian (female) duct. We characterized an androgen-dependent gene product from the mouse seminal vesicle (Wolffian duct derivative and *male* "natural reporter gene") and an estrogen-dependent gene product from the mouse uterus (Müllerian duct derivative and *female* "natural reporter gene"). We chose specialized secretory proteins from each tissue to characterize.

Androgen-dependent secretory proteins had been characterized from the rat seminal vesicle (26). These seminal vesicle secretory (SVS) proteins were named for their migratory position on acrylamide gels as SVS-IV, V and VI. We identified and characterized an SVS-IV from the mouse seminal vesicle (27). In both the rat and mouse, SVS-IV was under control of androgen and, therefore, not expressed in castrate male or female tissues.

In the case of a female natural reporter gene, we characterized an estrogen-dependent uterine

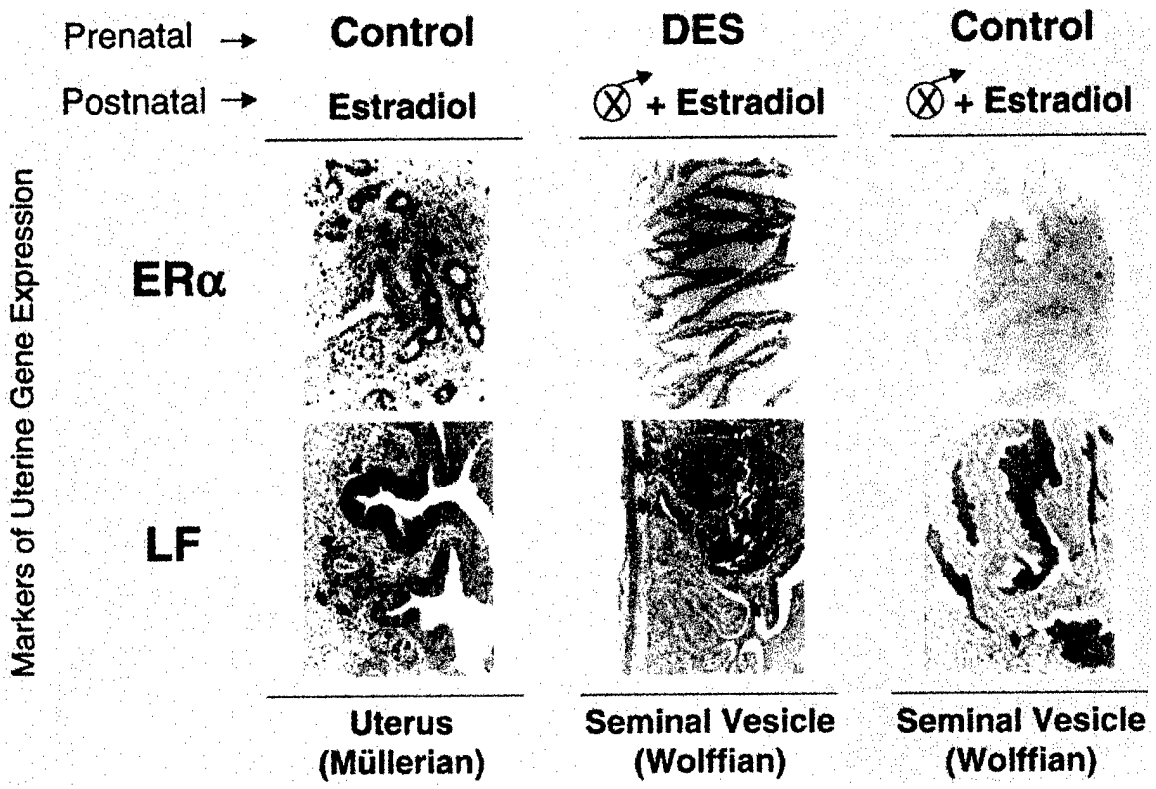

Fig. 3. Expression of male and female genes in the seminal vesicle of mice exposed prenatally to DES. Example of molecular feminization that accompanies Developmental Estrogenization. ERα=estrogen receptor α, LF= lactotransferrin.

secretory protein (28). The gene encoding the secretory protein was identified as lactotransferrin, a member of the transferrin gene family (29). Lactotransferrin was under powerful control by estrogen, located in the uterine epithelium, and varied with estrogen levels during the estrous cycle (30). Lactotransferrin was expressed in mammary gland, and leukocytes, but was not apparently regulated by estrogen in these tissues (30).

In control male mice, lactoferrin was not detected in the seminal vesicle; control mice that were castrated and treated with estrogen did not express lactoferrin (31). This was not unexpected, since we had shown that lactoferrin was primarily expressed and under estrogen control in the female reproductive tract. Moreover, the seminal vesicle of mice is derived exclusively from the male genital tract anlagen, the Wolffian duct, unlike the prostate gland, which is formed by the Wolffian duct, but also components of the urogenital sinus as well as the female anlagen, Müllerian duct.

Molecular feminization of the developmentally estrogenized male

When the seminal vesicles of prenatally DES-exposed males were analyzed, lactoferrin mRNA was detected. The level of lactoferrin message expressed increased following castration of the male and exceeded that of the uterus when each was estrogen stimulated. These results comprise the first demonstration of hormonally altered sexual development at the gene level (see Fig. 3 for summary).

Further analysis of the seminal vesicles of DES-treated mice demonstrated that while they retained the cytoarchitecture associated with the male organ, they expressed epithelial gene products associated with the uterus, that is, lactoferrin and ERα (32). In fact, virtually all the cells of the DES-exposed mouse seminal vesicle epithelium express antigens recognized by antisera to lactoferrin and ERa.

Additional experiments were conducted to assess whether prenatal exposure to DES had feminized the seminal vesicle cells or, alterna-

tively, blocked the masculinization of the organ at the molecular level. We were able to show that DES-exposed mouse seminal vesicles were competent to express the seminal vesicle specific protein, SVS-IV, under androgen control as well as lactoferrin. In fact, the same cell was often able to make both products (33).

Estrogen-associated DNA methylation in molecular feminization

The actual mechanism underlying the molecular feminization of genes by estrogen is still not elucidated. However, other studies in our laboratory on the control of lactoferrin expression may prove informative. We showed that developmental exposure to estrogens resulted in the persistent overexpression of lactoferrin in the uterus of females (34). The cellular secretory protein is expressed at the mRNA and protein levels as if the mouse is receiving injections of estrogen, even 3 weeks following ovariectomy. More recently, we have shown that the persistent expression of a normally hormone-regulated gene may be the result of imprinting (35). A fundamental event in cell differentiation involves programming genes to be differentially regulated later in life. One mechanism for developmental gene programming is selective DNA methylation or demethylation of its promoter. Our experiment involved analyzing the methylation pattern of the lactoferrin gene promoter from the uteri of mice that were exposed developmentally or as adults to DES. Our results demonstrate that developmental exposure to DES leads to a persistent change in the methylation pattern of the lactoferrin gene promoter, while adult exposure does not. Moreover, the former treatment results in persistent expression of the gene, while the latter does not. Thus, imprinting of genes by estrogens during critical periods of development may be both important mechanisms for functional defects later in life as well as a sensitive marker of exposure.

POTENTIAL GENE TARGETS FOR DEVELOPMENTAL ESTROGENIZATION SYNDROME

While estrogen-related imprinting of the lactotransferrin gene in male mice exposed in utero to DES provides the first useful model with which to conduct mechanistic studies, the functional significance of this change for the development of the male genital tract is not yet clear. Additional studies on developmental estrogenization of genes known to be involved in male genital tract development will be informative.

For example, Emmen et al. have recently shown that the cryptorchidism associated with prenatal treatment with various estrogens in mice may be the result of estrogen-related inhibition of an inter-organ regulatory factor, Insulin-like factor 3 (Insl3), production by the fetal testes (36). Insl 3 had been shown earlier to affect testicular descent through signaling from the fetal testes to a component of the genital mesentery, the gubernaculum. Insl3 mutant mice exhibit bilateral cryptorchidism; this is thought to occur through altered development of the gubernaculum, which retains an elongated "female" structure (37, 38).

Hypospadias, a defect of the external male genitalia associated with prenatal estrogen treatment, has also gained molecular dimensions recently (39). In this case, Yamada's group has reported that the development of the external genitalia of the mouse involves signaling by fibroblast growth factor (Fgf) during formation of the genital tubercle. Fgf 10 knock out mice show abnormal development of the glans penis, suggesting an important role for that signaling molecule in the induction of hypospadias.

Finally, the molecular mechanism for Müllerian duct retention associated with DES is becoming clearer. While it was shown some time ago that the effect of DES on Müllerian duct retention resides at the level of the duct, rather than the fetal testis (11), only recently has the molecular alteration in the duct been shown to be a failure of the MIS receptor in the fetal duct to respond to the peptide (40).

GENE IMPRINTING AND THE DEVELOPMENTAL ESTROGENIZATION SYNDROME PHENOTYPE

A general model has evolved over the last 30 years to explain the phenotype associated with Developmental Estrogenization Syndrome. Hormones and hormonally active chemicals act in a reversible fashion in which addition or withdrawal of the signaling stimulus results in

an increase and decrease of the response. This is indeed a physiological response system. However, in an immature or undifferentiated organism or cell, the response to a hormonal stimulus may be long lasting or irreversible. This then involves gene imprinting.

Gene imprinting by estrogenic compounds may arise through at least two mechanisms. In one case, the estrogenic chemical may directly imprint the gene through a process leading to persistent genetic change, probably at the level of DNA methylation. On the other hand, a biochemical memory could be formed by altering components of signaling pathways at key points in cell differentiation such that altered gene expression would ensue. The most likely genes to be important to this process would be those involved in response to secondary hormonal cues. Thus, when a gene programmed to respond to estradiol at puberty is misprogrammed or reimprinted by developmental exposure to a hormonally active chemical, it will respond abnormally to the secondary cue resulting in a functional cellular abnormality. This is the essence of disease (Fig. 4).

DES AND OTHER ESTROGENS IN THE ENVIRONMENT

The DES clinical findings added focus 30 years ago to the few reports of chemicals in the environment which mimicked estrogens. Among these were the findings that the pesticide DDT and some of its congeners were estrogenic, as was the related pesticide, methoxychlor. These chemicals were widespread in the environment, persistent, and synthesized without attention to their estrogen-like qualities.

On the other hand, DES, a rationally synthesized estrogen, was being introduced into the environment to accelerate growth in cattle. It was estimated that in 1971 alone, as much as 27,600 kilograms of DES was used in livestock feed lots (41). Based on model ecosystem studies done by Metcalf et al. one could expect that a certain amount of DES would find its way into the ecosystem (42). At that time, we (43) speculated that estrogens in the form of excreted oral contraceptives could reach the environment through the waste water system, adding to the overall estrogenic burden. Recently, studies of rivers in the UK have detected ethinyl estradiol, an estrogenic constituent of oral contraceptives, in water samples (44).

While interest in endocrine disrupting chemicals is currently widespread, there was a paucity of both interest and information 30 years ago. In fact, so little was known about the structural requirements for estrogenicity that the preface to the first Symposium on Estrogens in the Environment in 1979 included the following: "The objectives of the Symposium, stated quite simply, were to determine what an estrogen is and how it works, and what effect estrogenic substances might have on human health. These objectives seemed timely since many chemicals with diverse chemical structures, some of which are environmental contaminants, have been endowed with 'estrogenic' properties" (3).

The universe of chemicals "endowed with estrogenic properties" has grown enormously in the last 30 years. Environmental compounds have also been discovered that are anti-estrogens and anti-androgens. Species as diverse as snails and humans are thought to be affected by endocrine disruption. It is all the more remarkable that the principles used to establish this growing list of hormonally active chemicals were, and continue to be, based on the potent synthetic estrogen DES and the clinical experience first reported 30 years ago.

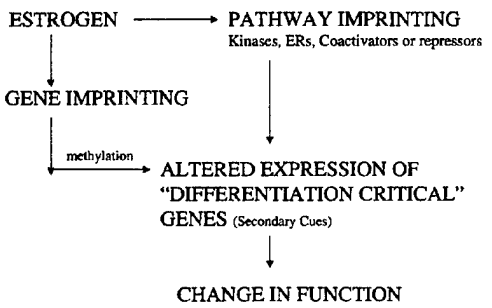

Fig. 4. Gene imprinting by hormones during development.

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ENDOCRINE DISRUPTION IN SEXUAL DIFFERENTIATION AND PUBERTY

What Do Pseudohermaphroditic Polar Bears Have To Do with the Practice of Pediatrics?

Cynthia C. Gulledge, PhD, Matthew E. Burow, PhD, and John A. McLachlan, PhD

What do pseudohermaphroditic polar bears and girls with premature breast development have in common? Hormones. Sexual differentiation and the initiation of secondary sexual characteristics, such as breast growth, are under the control of sex hormones, estrogen and androgen. Abnormal differentiation of the internal or external genitalia in bears and early onset of breast development in girls also may have a common element—exposure to environmental hormones. It has been shown that many common pesticides and environmental contaminants can act as estrogens or antiandrogens. Although there may be many factors contributing to sex-reversed animals or early pubescent girls, environmental hormone exposure is certainly one of the possible contributors.

Four years ago, Herman-Giddens et al[53] published a provocative article in which they reassessed onset of puberty in US girls and found that thelarche (Tanner stage 2B) occurs much earlier than the previously established norms and that menarche also occurs somewhat earlier, although the difference is not as drastic for this measure. Moreover, the percentage of girls less than 8 years of age with one or more secondary sexual characteristics (27.2% of black girls and 6.7% of white girls) is much higher than previously reported. This information is being viewed by some as a redefinition of normal, but others are calling it *precocious puberty* and sounding alarms about possible unnatural causes.

This study has caused a great deal of debate and led to a joint statement

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issued by the Endocrine Society and Lawson Wilkins Pediatric Endocrine Society in March 2001 calling for further research to define precocious puberty (www.endo-society.org/pubrelations/pressReleases/puberty.cfm) to determine whether girls really are reaching puberty earlier and, if so, to determine the cause. This reassessment in what is considered normal for hormonally associated physiologic change has many pediatricians searching for an answer: is it real? Some think that it is related to obesity, whereas others believe that a genetic component is primarily responsible. A recent preliminary report[57] on puberty and genes demonstrates a possible link. Within the context of an individual's genetic predisposition in conjunction with factors such as diet and obesity, what role does exposure to endocrine disrupting chemicals have on the normal physiologic process during puberty and reproductive development?

In addition to reports of possible increases in precocious puberty, other epidemiologic studies have described early isolated thelarche, globally decreasing sperm counts, and increased rates of congenital malformations of the male reproductive system (reviewed by Safe et al[79]). Information from studies on wildlife and in the laboratory is sufficient to associate endocrine disruption with the epidemiologic trends described in further detail later, but attributing causation remains difficult.

SOME ENVIRONMENTAL CHEMICALS ACT LIKE ESTROGENS

Many synthetic chemicals act as estrogen mimics (i.e., environmental estrogens). The heavy use of these compounds as pesticides and plasticizers, combined with their long half-lives, make them ubiquitous contaminants in the environment. One of the best-known examples is the pesticide DDT. Although the intended function of DDT was far removed from that of a vertebrate sex hormone, it was shown as far back as 1950 to cause estrogen-like effects in animal models (reviewed by Kupfer[63]). Environmental estrogens are not nearly as potent as are endogenous or pharmaceutic estrogens but are slower to degrade and can accumulate in lipid stores.[62, 93] Their ability to bioaccumulate in individuals and biomagnify up trophic levels may allow them to elicit significant estrogenic effects in hormone-responsive tissues. In some cases, the metabolites are more estrogenic than the parent compound.[62, 73]

This review focuses on estrogenic effects of environmental contaminants, but other endocrine-disrupting modes of action have been documented and should be considered when reproductive system anomalies are observed. No strong androgen mimics have been identified, but many environmental estrogens are also antiandrogenic (e.g., bisphenol A, o,p'-DDT, and methoxychlor) in yeast assay, and some fungicides (e.g., vinclozolin) are antiandrogens but are not estrogenic.[59, 60, 85] The possibility exists that some environmental compounds also display antiestrogenic activity (e.g., methoxychlor metabolites) by direct action at estrogen receptor (ER)-β (but not ER-α),[34, 35] whereas some (e.g., the fungicide fenarimol) can have this action indirectly by inhibiting aromatase conversion of testosterone (T) to 17β-estradiol (E2).[54] Other possible modes of action include those involving thyroid hormone metabolism (e.g., dioxin) and progesterone activity.[8, 13, 61] Lethal effects traditionally were the standard for determining safe levels of chemicals in the environment, but now there is recognition that sublethal effects, particularly on the reproductive system, could impact wildlife and human populations.[19, 93]

EFFECTS SEEN IN THE WILD

Reproductive anomalies potentially attributable to environmental estrogen exposure have been documented in many wildlife species (Table 1), including cases of intersex fish, malformations of external genitalia in alligators, reproductive impairment in fish-eating birds, reproductive failure in seals (reviewed extensively by Tyler et al[93] and Vos et al[94]), and pseudohermaphroditism in polar bears.[96] Because many environmental estrogens enter the environment through agricultural runoff and industrial effluent, it is not surprising that most examples of affected wildlife live in the water or eat aquatic organisms.

Intersex Fish

Vitellogenin (VTG) is a yolk protein precursor that is synthesized in egg-laying female fish only in response to estrogens, so it is a useful biomarker of estrogen exposure in male fish, which normally do not have high circulating levels of endogenous estrogen.[50] The presence of VTG in male fish has been demonstrated in marine and freshwater systems in the United States, United Kingdom, and Sweden (reviewed by Sumpter[88] and Vos et al[94]). In one study, VTG concentrations in male flounder from some British estuaries were extremely high (> 100,000 ng/mL) and often exceeded those normally found in sexually mature females.[1] Expression of VTG in a male fish indicates that the liver is currently exposed to estrogenic compounds; this could be the result of current exposure from the water (i.e., activational) or from effects in ovo that caused the differentiation of estrogen-producing ovarian tissue (i.e, organizational). In the study by Allen et al,[1] high VTG levels were accompanied in some fish by presence of an ovotestis (i.e., oocytes present in the testis), which suggests exposure during sexual differentiation, at least in those particular fish. Analysis of the water from sewage treatment effluents indicated the presence of not only some commonly found environmental estrogens (e.g., alkylphenols) but also detectable levels of natural estrogens and synthetic estrogens found in oral contraceptive pills.[22]

Malformations of External Genitalia in Alligators

After a major pesticide spill into Lake Apopka, Florida in 1980, juvenile male American alligators were found with abnormal gonads, depressed plasma testosterone concentrations, and small phallus size compared with those in a relatively uncontaminated control lake.[45, 46] Juvenile female alligators had elevated plasma E2 concentrations and exhibited abnormal ovarian morphology, with large numbers of polyovular follicles and polynuclear oocytes. Gonadal steroidogenesis in these juveniles was relatively unresponsive to stimulation with exogenous luteinizing hormone (LH). These data suggest that the gonads of juveniles from Lake Apopka were modified permanently in ovo, so that normal steroidogenesis was not possible. This speculation was supported indirectly by a recent study in which correlations of plasma levels of environmental estrogens with endogenous steroid concentrations or phallus size were not found, indicating that endocrine and morphologic abnormalities in the Lake Apopka alligators were probably organizational rather than dependent on current activational effects of contaminants.[47] Another study that expanded sampling to alligators from several other Florida lakes[48] found similar abnormalities

Table 1. EXAMPLES OF REPRODUCTIVE EFFECTS IN WILDLIFE POTENTIALLY CAUSED BY ENVIRONMENTAL ESTROGENS

Class	Organism	Environmental Estrogen/Source	Effect	Reference
Fish	Trout Flounder Carp	Sewage effluent containing natural and synthetic estrogen, alkylphenols	Vitellogenin (VTG) in males, intersex (ovotestis), decreased T and testicular growth, high VTG in females	1, 29, 50
Reptile	Alligator	Organochlorine pesticides	Low hatching rates, abnormal testes and ovaries, abnormal E2:T ratio, reduced phallus size, reduced gonadal response to LH	45, 46, 48
Bird	Gull Swallows	DDT; contaminated fish PCBs	Eggshell thinning, feminization, female–female pairing, abnormal nest building behavior	33, 67
Mammal	Polar bear	Contaminated seals, PCBs	Female pseudohermaphroditism, impaired immune system function	4, 21, 96
	Seal	PCBs, DDE, DDT, contaminated fish	Sterility, implantation failure, leiomyoma, adrenal hyperplasia, bone lesions	2, 72, 76
	Panther	DDE, PCBs	Feminization, cryptorchidism	26

LH = leuteinizing hormone.

in endocrine parameters and reproductive morphology. Although no major pesticide spills have been reported in these lakes, they are recipients of agricultural runoff and sewage treatment effluent and may have sufficient environmental estrogen contamination to produce these effects in ovo.

Reproductive Impairment in Fish-Eating Birds

Fish-eating birds (i.e., gulls) are high up on their food chain, so contaminants are biomagnified in their populations. The endocrine-disrupting effects of compounds such as DDT on embryos include impaired differentiation of the reproductive and nervous systems. The range of chemical effects on adult birds covers acute mortality, sublethal stress, reduced fertility, suppression of egg formation, eggshell thinning, and impaired incubation and chick-rearing behaviors (reviewed by Fry[33]).

Reproductive Failure in Seals

The population of common seal (*Phoca vitulina*) in the westernmost part of the Wadden Sea, the Netherlands, dropped from more than 3000 to fewer than 500 animals between 1950 and 1975, in part because of drastic reduction in pup production. Differences in reproductive success in the western and northern parts of the sea were correlated with tissue levels of polychlorinated biphenyls (PCBs) in the seals, related to feeding on fish from the more polluted western area.[76] Other seal populations (the Baltic grey seal, *Halichoerus grypus*, and ringed seal, *Phoca hispida*) suffered dramatic decreases in population during the 1960s and 1970s, also believed to be associated with exposure to environmental pollutants, such as PCBs and DDT.[52, 72] Some seals in these populations exhibited lowered reproductive capacity, uterine obstructions, leiomyomas, and adrenocortical hyperplasia. A recent report of results from postmortem examinations of Baltic grey seals between 1977 and 1996 indicates a decreased prevalence of uterine obstructions (from 42% to 11%) and leiomyomas (from 53% to 43%) and an increased prevalence of pregnancies (from 9% to 60%) when comparing the first and second decades of the study, corresponding to decreases in PCB and DDT contamination in the area of capture.[2] Although still not demonstrating causality, these studies show not only an increase in reproductive anomalies when contamination was elevated but also improvements in these same parameters when environmental conditions improved, providing stronger evidence for an endocrine disruption link.

Pseudohermaphroditic Polar Bears

Four female polar bears in Norway found to have male and female external genitalia, but no evidence of a Y chromosome, were classified as female pseudohermaphrodites.[96] Some of these bears had a penis containing a baculum (penis bone present in many mammalian species), whereas others exhibited clitoral hypertrophy. One suggested cause for their condition is hormonal dysfunction caused by environmental pollutants. PCBs have been detected at high concentrations in plasma of Norwegian polar bears, which, as top predators in their Arctic ecosystem, may biomagnify trace amounts present in lower trophic levels.[4, 21, 84] Because humans share top predator status and high-fat diets in common with

polar bears, they also may be at risk for pollutant biomagnification. Other similarities between polar bears and humans relevant to exposure in offspring include the degree of postnatal development required and the associated prolonged lactational period in the two species (reviewed by DeRocher[21]). Pregnancy and lactation are important processes in the off-loading of pollutants from mother to offspring.[75] During pregnancy, body burdens of organochlorines decrease by 23% for PCBs and up to 81% for DDT. Offspring transfer accounts for only 5% of the decline in DDT but as much as 66% of the decrease in PCBs (reviewed by DeRocher[21]).

In addition to the four pseudohermaphroditic bear cubs from the article by Wiig et al,[96] four other female pseudohermaphrodite polar bears have since been captured in the Svalbard, Norway area.[21] Female pseudohermaphroditism typically is caused by exposure to excess androgens, so it is not immediately clear how increased environmental estrogen load could cause this effect. As mentioned earlier, many environmental "estrogens" actually have effects on multiple endocrine pathways, and these are currently under investigation in the Svalbard polar bear population[84] (DeRocher, personal communication, 2001).

EFFECTS SEEN IN THE LABORATORY

One criticism of the wildlife studies is that relationships between effects seen in wildlife and high concentrations of contaminants in tissues or in the local environment are only correlative.[78] Demonstrating a causal link requires exposing animals to suspected endocrine disruptors in a controlled laboratory setting.[94] Laboratory studies indicate that prenatal exposure to these chemicals (especially during sexual differentiation) produces the most pronounced effects on morphology, endogenous hormone levels, and behavior, although exposure in prepuberty[39, 86] or adulthood[9, 41] also has consequences (Table 2).

To complement the findings in the wild, some studies have been conducted on wildlife species in captive or semicaptive conditions that demonstrate organizational or activational effects of environmental estrogen exposure. A recent study describes complete, permanent, and functional male-to-female sex reversal in the Japanese medaka fish (*Oryzias latipes*, d-rR strain) after a onetime embryonic exposure to o,p'-DDT during the crucial period for sex determination.[23] Organizational effects also were seen after injection of DDT into gull eggs at environmentally relevant concentrations, which induced abnormal development of an ovotestis and retention of müllerian ducts in male embryos.[32] Laboratory studies on domestic ducks have demonstrated that the effect of DDT on eggshell thinning is achieved by its metabolite (DDE) on metabolism of calcium and prostaglandin in the eggshell gland mucosa.[64] Another laboratory study demonstrating activational effects involved exposure in the laboratory of male rainbow trout (*Oncorhynchus mykiss*) to four different environmental estrogens, which caused a dose-dependent increase in VTG expression that was accompanied by a decrease in testis size.[56] Some other studies fall in between field and laboratory, such as one in which female seals were fed fish from contaminated sites or relatively clean sites. Females fed contaminated fish had lower reproductive success and lower E2 levels.[76] Polar bear studies currently underway include immunizing bears from Svalbard (high pollution) and Hudson Bay, Canada (relatively low pollution) with vaccines and drawing blood 5 weeks later to test immune response and determine whether immunity is compromised in bears with higher PCB loads.[21]

Laboratory studies using rodents provide a link between effects of environ-

Table 2. EXAMPLES OF REPRODUCTIVE EFFECTS CAUSED BY ENVIRONMENTAL ESTROGENS IN THE LABORATORY

Organism	Environmental Estrogen	Exposure	Effect in Male	Effect in Female	Reference
Fish	DDT	In ovo	Male-to-female sex reversal		23, 56
	Alkylphenols	Adults	↓ testis size, ↑ VTG		
Frog	DBP	Tadpoles	Complete or partially developed ovaries		71
Gull	DDT	In ovo	Feminization, ovotestis, retained müllerian ducts		32
Rodent	DES	In utero (transplacental)	Retained testes, epididymal cysts, distended seminal vesicles, retained müllerian ducts, abnormal sperm, reduced fertility, increased testicular cancer	Vaginal cornification subfertility paraovarian cysts	40, 68, 79
	OP		↓ testis wt., ↓ # sperm	Advanced vaginal opening (early puberty?)	
Rodent	DES (injected)	Early postnatal	↓ basal and LHRH-induced release of LH	↓ basal and LHRH-induced release of LH	25, 80, 81
	DDT (injected)		↓ basal and LHRH-induced release of LH		
	PCB (translactational)		abnormal mating behavior ↓ prostate and ↑ testis weight	Delayed vaginal opening, ↓ uterine weight, irregular estrus, pregnancy failure, ↓ uterotrophic effect of E2	
Rodent	DES DBP	Prepuberty or adulthood		Advanced vaginal opening, ↑ mitotic activity and mass of uterus in ovx females, pregnancy failure	9, 38, 39, 41, 42, 69, 86
	OP		↓ LH, ↓ FSH, ↓ T, ↑ PRL		
	MXC		↓ sperm count, ↑ pituitary PRL, ↓ behavioral latency (mounting, ejaculation), delayed puberty, ↑ time to pregnancy of mate	↑ wheel running advanced vaginal opening advanced first estrus persistent vaginal estrus	

DBP, dibutyl phthalate; DES, diethylstilbestrol; MXC, methoxychlor; OP, octylphenol; VTG, vitellogenin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHRH, luteinizing hormone–releasing hormone; ovx, ovariectomized; PRL, prolactin; T, testosterone.

mental estrogens seen in free-ranging or captive wildlife and potential effects for humans. In 1975, the authors[68] described a suite of structural defects in male CD-1 mice exposed prenatally to diethylstilbestrol (DES), a potent synthetic estrogen receptor agonist. These abnormalities in the reproductive system included retained testes (cryptorchidism), epididymal cysts, distended seminal vesicles, and retained müllerian ducts. The animals also were shown to have abnormal sperm, reduced fertility, and increased testicular cancer. The severity of these changes was dose-dependent, as were the appearance of all the lesions in the suite.

The DES model provides evidence for effects of potent estrogen receptor agonists on the developing reproductive system, but does this really reflect possible effects of weakly estrogenic chemicals present at low doses? Other studies have addressed this point by dosing rodents in utero or postnatally with the environmental estrogens in question (Table 2). The timing of exposure (e.g., prenatal versus postnatal) to estrogenic chemicals can account for many differences in end results. Prenatal exposure, particularly during crucial periods for sexual differentiation, is more likely to cause morphologic abnormalities matching those seen after in utero exposure to DES. Prepubertal (between weaning and puberty) exposure, on the other hand, is not likely to lead to abnormalities in gross morphology because it is outside of the crucial window for sexual differentiation but has been shown to impact reproductive behavior, circulating hormone levels, and the timing of the onset of puberty.[9, 38, 39, 41, 42, 86]

Sex-specific effects of estrogens on timing of puberty onset, as noninvasively measured by day of vaginal opening in female rats and preputial separation in male rats, have been observed (Table 2). In many cases, estrogenic chemicals advance the onset of puberty in females[39] and delay it in males.[86] Extrapolating results regarding puberty onset in rats to possible effects in humans may be complicated by evidence that central neuroendocrine mechanisms regulating puberty onset may differ between rats and humans. As described later, onset of puberty in humans seems to be mediated by release of central inhibition of LH-releasing hormone (LHRH) secretion, but in rats this may require direct stimulation of LHRH neurons rather than release from inhibition (reviewed by Terasawa and Fernandez[90]).

SEXUAL DIFFERENTIATION AND PUBERTY ONSET IN HUMANS

Normal sexual differentiation and the developmental, growth, and reproductive changes during puberty are clearly a function of the endocrine system driven by an intrinsic genetic program.[43, 44] Understanding normal and pathologic events related to sexual differentiation and puberty onset provides some perspective for demonstrating where environmental estrogen exposure could play a role in disrupting these processes.

Sexual Differentiation

The similarity in the morphogenesis of the reproductive system in mammals as diverse as mice and humans provides comparative insights into the spectrum of effects associated with in utero estrogen exposure in either. Mice and human fetuses progress from an "indifferent" stage of internal genitalia, in which the presumptive male (wolffian duct) and female (müllerian duct) reproductive

organs coexist regardless of the genetic sex of the fetus, to the definitive structure of the appropriate gender. Similarly, external genitalia develop in a sex-specific fashion from a single, indifferent state. This process is under the control of hormones from the fetal testes after differentiation of the fetal gonad, which is stimulated by at least one gene (e.g., *SRY* in mammals) on the Y chromosome. The configuration is female unless the fetal testis intervenes by secreting müllerian-inhibiting substance (MIS) to induce regression of the müllerian duct and testosterone to maintain the wolffian duct and masculinize external genitalia. This process can be illustrated with a pathologic example: when genetic females are exposed to abnormally elevated androgen levels (e.g., because of congenital adrenal hyperplasia) during the crucial period for sexual differentiation of external genitalia, they are partially masculinized, with enlarged clitoris and labia resembling a scrotum.[43]

In 1975, the same year as the authors' report of the effects of prenatal exposure to DES on the male offspring in mice,[68] studies were published in which genital defects also were observed in men whose mothers had taken DES during pregnancy, primarily to avoid miscarriage.[5, 6] These DES-exposed men had a higher prevalence of undescended (cryptorchid) testes and epididymal cysts than did comparable unexposed men. Follow-up studies showed, in addition, a higher prevalence of hypoplastic testes and abnormal sperm.[7, 37]

Onset of Puberty

The timing of puberty onset is controlled by neuroendocrine pathways that are organized during gestation.[44] Although negative feedback of sex steroids on the hypothalamic-pituitary-gonadal (HPG) axis is operational in both sexes by late gestation, positive feedback mechanisms that allow elevated estradiol to cause an ovulatory LH surge in females are not mature until late puberty. Gonadal sex steroids are released in infancy, when the primary negative control of the HPG axis seems to be gonadal–steroid dependent, but then, during childhood, LHRH secretion is suppressed centrally by a gonadal–steroid independent mechanism, preventing the onset of puberty (although low amplitude LHRH pulses and low concentrations of gonadal steroids are present during this time). Release of that central inhibition allows for increased amplitude of LHRH pulsatile release, which is accompanied by increased responsiveness of the pituitary to LHRH and of the gonad to LH and follicle-stimulating hormone (FSH), followed by the associated pituitary and gonadal responses that mark the beginning of puberty. Neural substrates and mechanisms controlling the timing of puberty onset are not well understood but are speculated to involve a "master gene" for the release of central inhibition.[90] One factor that plays at least a permissive role for onset of puberty in girls is body condition; girls with low body fat tend to have delayed puberty onset, and obese girls tend to enter puberty on the early side of normal.[44]

An early sign of puberty is breast development, which, in humans, is triggered by an increase in the ratio of estrogen to androgen. Adrenarche (i.e., an increase in release of adrenal androgens), which seems to be independent of changes in the HPG axis, occurs in boys and girls and is responsible for the appearance of axillary and pubic hair.[44] A recent preliminary report describes an association between homozygous presence of alleles for a high activity variant of the CYP3A4 enzyme (CYP3A4*1B) responsible for testosterone hydroxylation and onset of breast development.[57] Of girls (aged 9.5 ± 0.3 years) found to be homozygous for CYP3A4*1B, 90% exhibited breast development (Tanner score

2B or higher), whereas only 40% of girls homozygous for the low-activity variant (CYP3A4*1A) exhibited breast development at the same age. Removal of testosterone would increase the estrogen-to-androgen ratio, perhaps explaining why girls with increased testosterone hydroxylation would begin breast development earlier than girls expressing a lower-activity enzyme. Other means of increasing the estrogen-to-androgen ratio, such as increased estrogen caused by overexpression of aromatase (the enzyme that converts T to E2), have been shown to cause premature breast development in girls and gynecomastia in boys.[12, 87]

THE ENDOCRINE DISRUPTION HYPOTHESIS

Studies in wildlife point to the possibility that exposure to environmental estrogens is disrupting reproductive system development and function, a hypothesis that is bolstered by evidence from controlled studies in the laboratory and also the model system of DES exposure in rodents. That same model system produced effects in rodents that mimic effects seen in humans exposed to DES in utero, indicating that inappropriate estrogen exposure disrupts normal reproductive system development in humans as in rodents, the effects of which may not be evident until long after exposure. Administering estrogens or environmental estrogens prenatally or prepubertally can alter the timing of puberty in rodents. These observations led to the hypothesis that estrogenic chemicals in the environment may be affecting reproductive system development or the timing of puberty in boys and girls.[15, 16, 30, 79] Evidence in support of this hypothesis has been reported in the epidemiologic studies detailed later (Table 3), but this idea also has received criticism.[78]

EVIDENCE

Epidemiologic Indicators

News of decreasing sperm density globally during 1938 to 1990[14] provoked widespread public interest and investigations into possible causes and the validity of the results (e.g., the article by Fisch and Goluboff[28]). A recent reanalysis that attempts to control for methodologic confounders and expands the data set to include 1934 to 1996 has supported the original results.[89] Others report increased rates of idiopathic hypospadias (urethral opening displaced toward the scrotum) and cryptorchidism (undescended testes),[20, 58, 66] although a recent meta-analysis of international data indicates that increases were limited to industrialized countries and may have leveled off in many of these countries around 1985.[74] If environmental estrogens are to blame for congenital malformations in male humans, the researcher might expect increased incidence in the more industrialized regions and leveling off of incidence after bans on the most potent compounds (e.g., DDT) and improved surveillance. For most studies, there are few or no data on environmental estrogen exposure or tissue loads. One exception is a study from Spain, in which cryptorchidism rates at local hospitals were correlated to estimated pesticide exposure based on place of residence; rates tended to be higher in areas with intensive farming.[36] Increased risk for cryptorchidism (but not hypospadias) also was detected in sons of women (but not men) employed in the gardening industry, indicating a possible association with exposure to pesticides in utero.[95] Testicular cancer rates have increased in several

Table 3. REPRODUCTIVE ANOMALIES IN HUMANS POTENTIALLY CAUSED BY ENVIRONMENTAL ESTROGEN EXPOSURE

Observation	Environmental Estrogen	Other Possible Explanations	References
Isolated early thelarche	Phthalate esters, estrogens in hair care products and diet (e.g., poultry)	Mistaken assessment of fatty tissue, obesity, leptin, inactivity, maternal hormonal imbalance while in utero, phytoestrogens in soy infant formula, homozygous alleles for *CYP3A4*1*	17, 31, 53, 57, 70, 77, 92
Precocious puberty	—	Trend not real, improved nutrition and health, obesity, homozygous alleles for *CYP3A4*1*	53, 57
Decreased sperm count	—	Trend not real (methodologic differences), lifestyle	28, 83, 89
Increased rates of cryptorchidism	Pesticides, occupational exposure of mothers (gardeners)	Trend not real	20, 36, 66, 74, 95
Increased rates of hypospadias	—	Trend not real or not increasing since 1985, changes in minimum criteria to diagnose	20, 58, 66, 74
Increased rates of testicular cancer	—	Improved diagnosis	3, 24

countries over the past several decades,[3, 24] but the upward trends have been related inversely to DDE concentrations in breast milk,[24] so other environmental estrogens, if any, may be responsible. Testicular tumors have been detected in mice after DES exposure in utero,[68] and reductions in sperm count can occur after in utero or prepubertal exposure to endocrine disrupting chemicals (e.g., DES, octylphenol, or methoxychlor).[42, 68] The congenital malformations are similar to those seen in mice and humans exposed to DES in utero[37, 68] and raise the possibility that in utero exposure to endocrine disrupting chemicals may be contributing factors.

What about girls? Much attention lately has focused on the age of onset of puberty in girls. For decades, expected time for onset of puberty has been based on normal values established by Marshall and Tanner[65] using a British study population. The Herman-Giddens study[53] reassessed onset of puberty in US girls (N = 17,077) and found that thelarche (Tanner stage 2B) occurs much earlier (mean age, 8.87 years and 9.96 years for black and white girls, respectively, compared with 11.2 years[65]) than the previously established normal values and that menarche also occurs somewhat earlier (12.16 years and 12.88 years compared with 13.5 years), although the difference is not as drastic for this measure. Moreover, the percentage of girls less than age 8 years of age with one or more secondary sexual characteristics (27.2% of black girls and 6.7% of white girls) is much higher than previously reported. Whether these changes reflect a redefinition of normal or could be the result of environmental influences are matters of debate. In addition, because the striking changes in age of onset are seen in breast development or pubic hair appearance rather than menarche, it is unclear whether these results reflect a true change in puberty onset (suggesting a release of central inhibition of LHRH secretion) or increased prevalence of isolated premature thelarche and adrenarche. Pelvic ultrasonography indicates increases in mean uterine (> 1.8 mL) and ovarian (> 1.2 mL) volumes in cases of early central precocious puberty but not in cases of isolated premature thelarche.[49] Comparisons between isolated premature thelarche and central precocious puberty may be especially important in the determination of what, if any, contribution is made by environmental agents. Laboratory studies on rodents showing that prepubertal exposure to environmental estrogens can advance puberty in females (e.g., that by Goldman et al[39]) raise the issue that environmental factors may play a role in the apparent change in puberty onset. A recent study of daughters born to women who had been exposed accidentally to polybrominated biphenyls (PBBs) found that menarche occurred earlier (mean, 11.6 years) in breastfed girls who also had exposure to high PBB levels in utero (> 7 ppb) than in breastfed girls exposed to lower levels in utero (mean, 12.6 years for low exposure, 12.2 years for moderate exposure) or in girls who were not breastfed (mean, 12.7 years), regardless of in utero PBB exposure.[10] These results suggest that postnatal (translactational) exposure to PBBs accelerated onset of puberty but that prenatal exposure had no discernible effect on this measure. Levels of some PBBs in breast milk have been increasing in Sweden over the past 25 years and possibly produce endocrine disrupting effects by disrupting thyroid hormone balance (reviewed by Hooper and McDonald[55]).

Several case studies of premature thelarche after known exposure (oral or transdermal) to estrogens (reviewed by New[70]) indicate that these prepubertal tissues are sensitive to exogenous estrogens. A survey of parents at four US Army pediatric clinics (N = 521) found that 64% of blacks (but only 6.9% of whites) use hair care products containing hormones or placenta, and that half of those parents also use the products on their children,[91] which may account for some racial differences in the Herman-Giddens study. A case study by the

author of the survey describes four black girls (aged 14–93 months) with premature thelarche or pubic hair that resolved after their mothers ceased using estrogen or placenta-containing hair care products on the girls, suggesting a transient (activational) effect of exogenous estrogen rather than a central neuroendocrine effect.[92]

The large number of cases of early thelarche seen in Puerto Rico in the 1980s still provides an important model with which to assess environmental contributions to advancing puberty in girls.[11, 18, 77] Girls aged 6 months to 8 years presented at Tanner stage 2B and above at much greater than expected rates, prompting the formation of the Premature Thelarche and Early Sexual Development Registry in Puerto Rico in 1988. Retrospective and prospective data from 1969 to 1998 have reported 4674 cases of premature thelarche on the island. Many explanations were proposed after analyses of endogenous hormones, diet, and environment, but none was definitive.[31, 77] Recently, studies from an early puberty cohort (aged 6 months to 8 years; mean, 31 months) in Puerto Rico showed a link between phthalate esters used as plasticizers and premature thelarche.[17] The endocrine disrupting mechanisms of phthalates include estrogenic and antiandrogenic actions.[51, 69] Increasing estrogenic activity or decreasing androgenic activity would increase the effective estrogen-to-androgen ratio, which may account for the connection between early thelarche and elevated plasma levels of phthalate esters in the Puerto Rican girls.

Possible environmental estrogen exposure sources for children in utero or postnatally include occupational exposure, industrial spills, plasticizers in plastic products, agricultural runoff and overspray,[27] diet (e.g., fish from contaminated waters or meat from animals treated with hormones), and estrogenic cosmetic products (e.g., hair care products[91] and sunscreen[82]). Certainly, the overall estrogenic hormone burden on the environment is larger than ever and increasing. In addition to the synthetic industrial chemicals mentioned earlier, there is an increased use of weakly estrogenic soy products in diets of newborn and prepubertal children, and the wastewater effluent has been shown to contain detectable levels of estrogens of human and agricultural origin. Because the sensitivity of children to hormonal perturbation is so great, the risk for untoward responses to these materials must be considered.

Caveats

Two recent reviews that agreed with the biologic plausibility of the environmental endocrine hypothesis also point out the current weaknesses they see in the linkage between environmental hormones and disease in wildlife[93] and humans.[78] In some cases, the secular health trends with which environmental endocrine-disrupting chemicals are associated are themselves debated or disputed. The underlying science often moves as fast or faster than the testable health hypotheses.

WHAT DOES ALL THIS MEAN TO PEDIATRICIANS?

Because so much of the material is so new and experimental, concrete recommendations are difficult to make. The clinician may compare the health–science interaction related to estrogens in the environment to the early days of lead research and health recommendations. It took years of research and debate to establish lead–health links and to set standards. The environmental hormone–

health links are still to be made. Although the research goes on, pediatricians will benefit by knowing that:

1. There are hormonally active environmental chemicals that may be affecting the sexual development of many species.
2. It is likely that pregnant women and prepubescent children are exposed to some of these materials.
3. Alterations in estrogen–androgen levels or exposure to exogenous estrogens in fetuses and children have predictably deleterious effects on puberty, breast growth, and development of the internal and external genitalia.

Rather than causing undue concern among their patients, pediatricians can use this knowledge as a means toward understanding possible environmental contributions to disorders for which genetic or other etiologic explanations are not readily apparent. Conversely, pediatricians can play an important role in supporting or disproving the hypothesis that environmental estrogens or antiandrogens play a role in human reproductive disorders manifested in newborn infants and older children. The biologic plausibility of the hypothesis compels the field of medicine to seek its answer.

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CURRICULUM VITAE

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EDUCATION AND TRAINING

1998 – 2000 **Post-Doctoral Fellow**, Laboratory of Molecular and Environmental Endocrinology, Center for Bioenvironmental Research at Tulane and Xavier Universities, New Orleans, Louisiana. Mentor: John A. McLachlan

1994 – 1998 **Ph.D.** Program in Molecular and Cellular Biology,
Tulane University. New Orleans, LA
Dissertation Title: "Molecular mechanisms of survival signaling and suppression of apoptosis in human breast carcinoma cells."
Advisor: Barbara S. Beckman Ph.D.

1989 – 1994 **B.S.** Biological Sciences: Molecular Biology emphasis
University of Southern Mississippi, Hattiesburg, MS.

PROFESSIONAL EXPERIENCE

4/02-Present **Assistant Professor**, Section of Hematology & Oncology, Department of Medicine, Tulane University Health Sciences Center.

4/02-Present **Adjunct Assistant Professor**, Department of Surgery, Tulane University Health Sciences Center.

4/02-Present **Program Member**, Tulane Cancer Center, Tulane University Health Sciences Center

4/00 – 4/02 **Research Assistant Professor**, Department of Pharmacology, Tulane University Health Sciences Center, New Orleans, Louisiana

AWARDS AND HONORS

1995-1997	National Institutes Of Health Graduate Pharmacological Sciences Training Grant Fellowship. Dept. of Pharmacology, Tulane University.
1996	Cancer Association Of Greater New Orleans Student Research Grant
1997	Morris F. Shaffer Award For Excellence In Student Presentation, Tulane University.
1998	Tulane Cancer Center Student Travel Award, to attend AACR conference, Molecular Mechanisms of Apoptotic Regulation
1998	Eleventh Annual Morris F. Shaffer And Margaret H.D. Smith Shaffer Award For Excellence In Research. Tulane University.
1999	Post-Doctoral Travel Award, Hormonal Carcinogenesis Gordon Research Conference

PROFESSIONAL SOCIETIES AND ACTIVITIES

1993-present	Member, American Association for the Advancement of Science.
1998-present	Member, American Association for Cancer Research
2000-present	Associate Member, Endocrine Society
2001-present	Member, American Chemical Society
1999	Reviewer, Cell Growth and Differentiation.
1999-2001	Reviewer, Cancer Research
2000	Reviewer, Journal of Neurochemistry
2000	Reviewer, Histology and Histopathology
2001	Reviewer, Toxicological Sciences

TEACHING EXPERIENCE

1994-1995	**Instructor**, General Biology Laboratory (CELL 101) Department of Cell and Molecular Biology, Tulane University
1996-1998	**Lecturer**, "Cancer biology and chemotherapeutics" in, Drugs and their Actions (GPHR605/CELL305/CHEM305) Department of Pharmacology, Tulane University
1997-2000	**Lecturer**, "Signal Transduction, the JAK-STAT pathway" in, Mechanisms of Hormone Action (Graduate Course: GPHR 756). Departments of Pharmacology and Physiology, Tulane University Health Sciences Center
2000	**Lecturer**, "Apoptosis in Cancer" in, Molecular Basis of Cancer (Graduate course: ENHS 781). Department of Environmental Health Sciences, Tulane University Health Sciences Center

PUBLICATIONS

Burow, M.E., Weldon, C.B., Tang, Y., Navar, G.L., Krajewski S., Reed, J.C., Hammond, T.G., Clejan, S. and Beckman, B.S. Differences in Susceptibility to Tumor Necrosis Factor-α-Induced Apoptosis Among MCF-7 Breast Cancer Cell Variants. *Cancer Research* **58**: 4940-4946 (1998).

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Burow, M.E., Weldon, C.B., Melnik, L.I., Duong, B.N., Collins-Burow, B.M., Klippel, A., Beckman, B.S., McLachlan J.A. Phosphatidylinositol 3-kinase/Akt mediated regulation of NF-κB signaling events as a mechanism for suppression of TNF-induced apoptosis. *Biochemical and Biophysical Res. Comm.* **271**: 342-345, (2000).

Burow, M.E., Weldon, C.B., Chiang, T-C., Tang, Y., Collins-Burow B.M., Rolfe, K., Li, S., McLachlan, J.A., Beckman, B.S. Differences in protein kinase C and estrogen receptor α, β expression and signaling correlate with apoptotic sensitivity of MCF-7 breast cancer cell variants. *Int. J. Oncol.* **16**: 1179-1187, (2000).

Alam, J., Dunne, C., Stewart, D., Touchard, C., Otterbein, S., Choi, A.M.K., **Burow, M.E.,** Tou J-S. Mechanism of Heme Oxygenase-1 Gene Activation by Cadmium in MCF-7 Mammary Epithelial Cells. *J. Biol. Chem.* **275(36)**: 27694-27702 (2000).

Collins-Burow, B.M., **Burow, M.E.,** Duong, B.N., McLachlan, J.A. The estrogenic and anti-estrogenic activities of flavonoid phytochemicals through estrogen receptor binding dependent and independent mechanisms. *Nutrition and Cancer.* **38(2)**, 229-244 (2000).

Burow, M.E., Boue, S., Collins-Burow, B.M., Melnik, L.I., Duong, B.N., Li, S.F., Wiese, T., Cleavland, E., McLachlan J.A. Phytochemical glyceollins, isolated from soy, mediate anti-hormonal effects through estrogen receptor alpha and beta. *J. Clin. Endocrinol. and Metabolism* **86(4)**, 1750-1758, (2001).

McLachlan, J.A., **Burow, M.E.,** Chiang, T-C., Li, S.F. Gene Imprinting in Developmental Toxicology: A Possible Interface between Physiology and Pathology. *Toxicology Letters* **120**: 161-164, (2001).

O'Neil, J.S., **Burow, M.E.,** Green, A.E., McLachlan, J.A., Henson, M.C. Effects of estrogen on LEP gene promoter activation on MCF-7 breast cancer and JEG-3 choriocarcinoma cells. *Molecular and Cellular Endocrinology* **176(1-2)**:67-75, (2001).

McLachlan, J.A., Newbold, R.R., **Burow, M.E.,** Li, S.F. From malformation to molecular mechanisms in the male: three decades of research on endocrine disruption. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **109(4)**: 263-272, (2001).

Weldon, C.B., **Burow, M.E.,** Rolfe, K.W., Clayton J.L., Jaffe B.M., Beckman, B.S. NF-κB-mediated chemoresistance in breast cancer cells. *Surgery* **130(2)**:143-150, (2001).

Fox, J.E., Starcevic M., Kow, K.Y., **Burow, M.E.,** McLachlan, J.A. Nitrogen fixation: Endocrine disrupters and flavonoid signalling. *Nature* **413(6852)**: 128-129, (2001).

Gulledge, C.C., **Burow, M.E.,** McLachlan, J.A. Endocrine disruption in sexual differentiation and puberty: What do pseudohermaphroditic polar bears have to do with the practice of pediatrics? *PEDIATRIC CLINICS OF NORTH AMERICA* **48(5)**: 1223-1240, (2001).

Fox, J.E., **Burow, M.E.,** McLachlan, J.A. Symbiotic gene activation is interrupted by endocrine disrupting chemicals. *TheScientificWorld* **1**: 653-655, (2001).

Burow, M.E., Weldon, C.B., Tang Y., McLachlan, J.A., Beckman, B.S. Oestrogen-mediated suppression of TNF-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. *J. Steroid Biochem. & Mol. Biol.* **78(5)**: 409-418, (2001).

Li, S.F., Ma, L., Chiang, T.C., **Burow, M.E.,** Newbold, R.R., Negishi, M., Barrett, J.C., McLachlan, J.A., promoter CpG methylation of *Hox-a10* and *Hox-a11* in mouse uterus not altered upon neonatal diethylstilbestrol exposure. *Molecular Carcinogenesis* **32**:213-219 (2001).

Frigo, D.E., Duong, B.N., Melnik, L.I., Schief, L., Collins-Burow, B.M., Pace, D.K., McLachlan, J.A., **Burow, M.E.** Flavonoid Phytochemicals Regulate Activator Protein-1 Signal Transduction Pathways in Endometrial and Kidney Stable Cell Lines. *Journal of Nutrition* (2002) in press.

Gozal, E., Ortiz, L.A., Zou, X., **Burow, M.E.,** Lasky, J.A., Friedman, M. Silica-induced Apoptosis in Murine Macrophage: Involvement of TNFα and NF-κB Activation. *American Journal of Respiratory Cell and Molecular Biology* (2002) accepted for publication.

MANUSCRIPTS SUBMITTED OR IN PREPARATION

Fox, J.E., Starcevic M., Jones, P.E., **Burow, M.E.**, McLachlan, J.A. Phytochemical signaling and symbiotic gene activation are interrupted by endocrine disrupting chemicals. Submitted to *Environmental Health Perspectives* (2001).

Frigo, D.E., **Burow, M.E.**, Mitchell, K.A., Chiang, T-C., McLachlan, J.A. DDT and its metabolites alter gene expression in human uterine cell lines through ER-independent mechanisms. Submitted to *Environmental Health Perspectives* (2001).

Weldon, C.B., Scandurro, A.S., Rolfe, K.W., Clayton J.L., Elliott, S.E., Butler, N.N., Melnik, L.I., Alam J., McLachlan, J.A., Jaffe, B.M., Beckman, B.S., **Burow M.E.** Identification of mitogen-activated protein kinase kinase 5 (MEK5) as a chemoresistance pathway in MCF-7 cells using gene expression microarray. Submitted to *Surgery* (2002).

Figueroa, Y.G., Chan, A.K., Ibrahim, R., Tang, Y., **Burow, M.E.**, Alam, J., Scandurro, A.S., Beckman, B.S. Mechanism of Erythropoietin gene activation by hypoxia in human hepatocellular carcinoma cells: Role for a phosphatidylinositol 3-kinase/AKT/NF-κB Signaling pathway. Revised submission to *Experimental Hematology* (2002).

Burow, M.E., McKee, A., Ramsey, N., Collins-Burow, B.M., Melnik, L., McLachlan, J.A., Beckman, B.S. Inhibition of phorbol ester mediated suppression of TNF-induced apoptosis through blockade of PKCα-MEK-AP1 signaling. A possible mechanism for the anti-tumor effects of apigenin. Under revision for resubmission to *Exp. Biol. Med.* (2001).

Duong, B.N., Frigo, D.E., Elliott, S., Weldon, C.B., Collins-Burow, B.M., Alam, J., Beckman, B.S., McLachlan, J.A., **Burow, M.E.** Crosstalk between Phosphatidylinositol 3-kinase/AKT and the estrogen receptor: a mechanism for regulating cell survival in MCF-7 breast carcinoma cells. In preparation for submission.

Duong, B.N., Elliott, S., Melnik, L.I., Tang, Y., Beckman, B.S., Alam, J., McLachlan, J.A., **Burow, M.E.** AKT regulation of ERβ-mediated transcription through AF-2 recruitment/activation of p160 Coactivators. In preparation for submission.

Burow, M.E., Collins-Burow, B.M., Frigo, D.E., Weldon, C.B., Elliot, S., Alam, J., McLachlan, J.A. Antiestrogenic activity of flavonoid phytochemicals mediated via c-jun N-terminal protein kinase and p38, Mitogen-activated protein kinase pathways. Isoform specific antagonism of estrogen receptor alpha. In preparation for submission.

Burow, M.E., Melnik L.I., Collins-Burow, B.M., Tang, Y., Elliott, S.E, Alam, J., Hill, S.M., Beckman, B.S., McLachlan, J.A. $G\alpha_{i2}$- and $G\alpha_o$-mediated regulation of estrogen receptors (α/β) via coactivator recruitment and activation. Manuscript in preparation for submission.

Collins-Burow, B.M., **Burow, M.E.**, Weldon, C.B., McLachlan, J.A. Induction of apoptosis by anti-estrogenic phytochemicals in breast carcinoma cells. Manuscript in preparation for submission.

PRESENTATIONS & INVITED LECTURES

Gordon Research Conference, Hormonal Carcinogenesis, 1999. Antiestrogenic Activity of Flavonoid Phytochemicals Mediated via c-Jun N-terminal Protein Kinase and p38 Mitogen Activated Protein Kinase Pathways.

Grand Rounds, Department of Pathology, Tulane University Health Sciences Center, Nov. 2000. Apoptotic/survival signaling in breast cancer cells. Role of the estrogen receptor and AKT.

Department of Structural & Cellular Biology and Tulane Cancer Center, Feb. 2001. Peptide Hormone Cross-talk with Estrogen Receptor Signaling in Breast Carcinoma Cells: A role for the phosphatidylinositol-3 kinase/AKT pathway.

Tulane Cancer Center, Tulane University Health Sciences Center, May. 2001. G-alpha-Protein Mediated Regulation of Estrogen Receptor Activation: Cell Signaling Pathways that Target the p160 Transcriptional Coactivators.

Department of Pharmacology & Experimental Therapeutics and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, September 2001. Cross-talk between G-protein and estrogen receptor signaling. Pathways that target transcriptional coactivators.

Hayward Humane Genetics Program, Tulane University Health Sciences Center, April 2002. "MEK5-BMK1/Erk5, a novel mitogen-activated protein kinase (MAPK) signaling pathway involved in regulation of apoptosis and drug-resistance of breast carcinoma cells."

Department of Biological Sciences, University of Southern Mississippi, April, 2002. "MEK5-BMK1/Erk5, a novel mitogen-activated protein kinase (MAPK) signaling pathway involved in regulation of apoptosis and drug-resistance of breast carcinoma cells."

ABSTRACTS

Burow, M.E., Tang, Y., Beckman B.S. Effects of environmental estrogens on apoptosis in MCF-7 breast cancer cells. Gordon Research Conference, Hormonal Carcinogenesis, 1997.

Burow, M.E., Weldon, C.B., McKee, A., Clejan, S., Beckman, B.S. Phorbol ester and insulin-like growth factor-I suppression of TNF-α-induced apoptosis through a phosphatidylinositol-3-kinase mediated inhibition of ceramide formation and caspase activation. Proceedings of the American Association for Cancer Research special conference on Molecular Mechanisms of Apoptosis Regulation, 1998.

Burow, M.E., Weldon, C.B., Tang, Y., Navar, G.L., Krajewski S., Reed, J.C., Hammond, T.G., Clejan, S. and Beckman, B.S. Differences in susceptibility to tumor necrosis factor-α-induced apoptosis among MCF-7 breast cancer cell variants. Proceedings of the American Association for Cancer Research, 1998.

Burow, M.E., Boue, S., Collins-Burow, B.M., Melnik, L., Duong, B.N., Li, S., Wiese, T., Cleavland, E., McLachlan J.A. Phytochemical glyceollins, isolated from soy, mediate anti-hormonal effects through estrogen receptor alpha and beta. E.Hormone 1999. (30^{th} anniversary of the 1^{st} estrogens in the environment meeting).

Burow, M.E., Melnik, L.I., Elliot, S., Collins-Burow, B.M., Alam, J., Hill, S.M., Beckman, B.S., McLachlan J.A. Galpha Protein-Mediated Regulation of Nuclear/Steroid Hormone Receptor Activation Through p160 Coactivator Targeting:. Gordon Research Conference, Hormonal Carcinogenesis, 2001.

GRANT AND CONTRACT SUPPORT (FUNDED- PENDING START DATE)

"Potential therapeutic use of glyceollins (I-III), novel anti-estrogenic flavonoid phytochemicals isolated from soy."
(Principal Investigator)
Agency: Department of Defense- U.S.A.M.R.M.C. (BC010983)
Type: **IDEA**, recommended for funding; $442,407
Using a nude mouse implanted breast carcinoma model, this proposal will investigate the possible chemopreventive and therapeutic activities of glyceollins (1-3), novel antiestrogenic flavonoids identified from elicited soy.

GRANT AND CONTRACT SUPPORT (ACTIVE)

"Coactivator and Corepressor Expression as a Mechansism for Regulation of Apoptosis and Cell Survival in Normal, Immortal and Neoplastic Breast Epithelial Cells"
(Principal Investigator)
Agency: Department of Defense- U.S.A.M.R.M.C.
Type: CONCEPT, **DAMD-17-01-1-0655**, Funded: May 15, 2001-April, 2002; $74,250
The primary objective of this proposal is the determine the function that expression levels of specific coactivator and co-repressor proteins have on regulation of apoptosis and cell survival in normal and malignant breast epithelial cells.

"The Role of Protein Kinases in HIF-1-Regulated Gene Expression"
Collaborative Investigator- (Principal Investigator: Dr. Barbara G. Beckman)
Agency: LA Cancer & Lung Trust Fund Board; 7/1/01-6/30/02
The primary long-term objective of this proposal is to understand the role of protein kinase C isoform specific signaling in regulation of HIF-1-mediated hypoxia responses and gene regulation.

"Role of mitogen activated protein kinases in control of the replication of a protozoan parasite, Toxoplasma gondii"
Co-Investigator-22% (Principal Investigator: Dr. Tyler J. Curiel)
Agency: J&J. Period: 3/02-3/05.

GRANT AND CONTRACT SUPPORT (COMPLETED)

"Effects of Environmental Estrogens on Apoptosis in Normal and Cancerous Breast Epithelial Cells"
Principal Investigator: Matthew E. Burow, Ph.D.
Agency: Department of Defense- U.S.A.M.R.M.C.
Type: DAMD17-97-1-7024. Period: 06/01/97-10/31/00, $61,334
The goals of this project were to identify specific environmental estrogens that suppressed apoptosis in breast epithelial cells and determine if these effects were mediated through increased expression of members of the Bcl-2 family of anti-apoptotic proteins.

"High Performance Liquid Chromatography for Quantitative Analysis of Environmentally Active Chemicals on Health, Disease, and Nutrition"
Co-written with- Principal Investigator John A. McLachlan
Agency: National Science Foundation.
Type: NSF Equipment Grant. Awarded: 8/15/99, $106,645
This equipment grant was written to obtain an HPLC-MS system to further the research goals of and provide critically need quantitative analysis capabilities for a group collaborating investigator driven projects focused on the examination of environmentally relevant endocrine disrupting chemicals in diet, health and disease.

GRANT AND CONTRACT SUPPORT (SUBMITTED)

"Melatonin/Estrogen Response Pathway in Breast Cancer"
Co-Investigator-10% (Principal Investigator: Dr. Steven M. Hill)
Agency: NIH-NCCAM
Type: **R01**, October 1, 2001;
This project looks at melatonin's modulation of the MAPK pathway (Erk1/2), and subsequent modulation of ERα transactivation --- via the melatonin G protein coupled Mel1a/mt1 receptor. This research will determine which G protein the Mel1a/mt1 receptor couples to, if it couples to multiple G proteins and how those signal to affect MAPK and ER.

"Parasite MAPK in T. gondii pathogenesis."
Co-Investigator-10%, (Principal Investigator: Dr. Tyler J. Curiel)
Agency NIH,Type: **R01** AI50824 Submitted March 01, 2002, $2,250,000.

"Ceramide and AKT/NFκB in Breast cancer Chemoresistance"
Co-investigator-10% (Principal Investigator: Dr. Barbara G. Beckman)
Agency: NIH-NCI (Experimental Therapeutics 1, ET1)
Type: **R01**, revised submission March. 01, 2002; $1,185,575
The long-term goal of this project will be to identify novel therapeutic strategies to reverse drug resistance in breast cancer through identification of specific molecular mechanisms by which the PKC/PI3K/AKT cascades regulate production and signaling through the sphingomyelinase-ceramide lipid pathway.